

## DNA Damage Repair Pathways in Cancer Stem Cells

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### Abstract

The discovery of tumor-initiating cells endowed with stem-like features has added a further level of complexity to the pathobiology of neoplastic diseases. In the attempt of dissecting the functional properties of this uncommon cellular subpopulation, investigators are taking full advantage of a body of knowledge about adult stem cells, as the "cancer stem cell model" implies that tissue-resident stem cells are the target of the oncogenic process. It is emerging that a plethora of molecular mechanisms protect cancer stem cells (CSC) against chemotherapy- and radiotherapy-induced death stimuli. The ability of CSCs to survive stressful conditions is correlated, among others, with a multifaceted protection of genome integrity by a prompt activation of the DNA damage sensor and repair machinery. Nevertheless, many molecular-targeted agents directed against DNA repair effectors are in late preclinical or clinical development while the identification of predictive biomarkers of response coupled with the validation of robust assays for assessing biomarkers is paving the way for biology-driven clinical trials. *Mol Cancer Ther*; 11(8); 1627–36. ©2012 AACR.

### Introduction

Mammalian cells have to constantly face genotoxic injuries due to exposure to endogenous and exogenous agents. Biochemical reactions generate reactive oxygen species (ROS) that avidly bind to nucleic acids. In addition, DNA chemical bonds physiologically undergo spontaneous degradation. DNA is also attacked by a variety of environmental mutagens of chemical, physical, and biologic origins (1). Given the heterogeneity of DNA-damaging agents and the correlated diversity of genetic lesions, eukaryotic cells exploit distinct, albeit partly overlapping, repair mechanisms (Table 1). In this manner, each repair avenue is engaged according to the type of lesion, even though the repair activity is carried out through a sequential recruitment of sensors, transducers, mediators, and effectors. The biologic outcome of DNA damage depends on several factors. For instance, a distinct response pattern characterizes different cell types, as shown by an extraordinary ability of adult stem cells to repair the genetic code compared with their offsprings (2). Additional determinants of cell fate are the extent of the lesion and the rapidity of its repair. Therefore, cell fate is

decided on the basis of the balance between tissue needs and damage severity. This is a process that culminates in a cellular response that can span from transient cell-cycle arrest to senescence induction or apoptosis.

DNA repair pathways encompass the nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), direct repair, and the double-strand break (DSB) recombinational repair. The NER pathway corrects bulky helix-distorting lesions caused by chemicals and ionizing radiations, the BER system targets small chemical alterations (base modifications), whereas MMR removes nucleotides mispaired arising from replication errors. The simplest repair mechanism is the monoenzymatic direct repair, a one-step methyl transfer reaction operated by the *O*<sub>6</sub>-methylguanine methyltransferase (MGMT) that defends cells against alkylating agent-generated lesions. The more challenging DSB repair is characterized by a different degree of fidelity in relation to the phase of the cell cycle. While the error-free homologous recombination repair (HRR) dominates in dividing cells, the G<sub>1</sub> phase acting nonhomologous end-joining (NHEJ) is error-prone, as a template for recombination is unavailable. These 2 pathways repair the majority of chemotherapy- and radiotherapy-induced damage. Finally, cell-cycle checkpoint components, such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia/Rad3-related kinase (ATR), and checkpoint kinases (Chk1 and Chk2), become engaged under replication stress or consequently to DSBs. These cell-cycle arrest mechanisms allow the recruitment of either DNA repair effectors or, when a cell is irreversibly damaged and the repair fails, proapoptotic molecules (3).

While these mechanisms preserve genome integrity by preventing transforming mutations, cancer cells improperly activate DNA repair pathways to overcome many standard anticancer treatments. This has fostered the

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**Table 1.** DNA repair pathways, target lesions, and diseases associated with DNA repair defects

DNA repair pathways	Pathway effectors	Target lesions/functions	Human disease associated with DNA repair defects
NER	XPA, XPB, XPC-RAD23B, XPD, XPE, XPF, XPG, RPA, TFIIH, ERCC1	Bulky and helix-distorting lesions	Xeroderma pigmentosum, Cockayne syndrome, trichothiodystrophy
BER	APE1, MUTYH, UNG, OGG1, NEIL1, NEIL2, NEIL3, NTHL1, MPG, TDG, SMUG1, POL $\beta$ , XRCC1, APTX, TDP1, PNKP, LIG1, LIG3, FEN1, PCNA	Base modifications	Cancer predisposition, neurodegenerative disorders, immunodeficiency
MMR	MSH2, MSH3, MSH6, MLH1, PMS2, EXO1	Sequence mismatches	Lynch syndrome (hereditary nonpolyposis colorectal cancer), Lynch syndrome variants, sporadic colon cancer, noncolonic tumors
MGMT pathway	MGMT (monoenzymatic pathway)	Alkylation (including methylation) at the O6 position of guanine	
HRR	BRCA1, BRCA2, RAD50, RAD51, MRN, XRCC2, XRCC3	DSBs- S-G <sub>2</sub> phase acting	Hereditary breast ovarian cancer syndrome
NHEJ	KU70, KU80, XRCC4, DNA-PKc, DNA ligase IV	DSBs- G <sub>1</sub> -S phase acting	Syndromes with brain development defects and immunologic abnormalities
Fanconi anemia pathway	FANCA, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCI, FANCL, FANCM, FANCN	Interstrand DNA cross links	Fanconi anemia
ATM/ATR-mediated signaling	ATM, ATR, CHK1, CHK2, MRN, ATRIP, MDC1, 53BP1, MCPH1/BRIT1, RNF8, RNF168/RIDDLEIN, RAD17, RAD9-RAD1-HUS1 (9-1-1) complex	Cell-cycle checkpoints	Ataxia-telangiectasia, Nijmegen breakage syndrome

development of DNA damage pathway-interfering agents, and many of these compounds are undergoing clinical trials. In such a scenario, the functional characterization of cancer stem-like cells endowed with multiple protective mechanisms (4), even including an extreme proficient DNA repair machinery, is crucial for sharpening the therapeutic potential of these compounds.

### DNA Repair in Adult Stem Cells

Adult stem cells are a dedicated pool of undifferentiated cells that maintain tissue homeostasis by counteracting cell loss due to physiologic cellular turnover or tissue injuries. To do this, stem cells have the unique capacity to self-renew through which they give rise, at

each division, to a daughter cell that retains the parental phenotype to avoid depleting the original pool and a second cell that differentiates to finally acquire tissue-specific functions. Because stem cells ensure the lifetime function of tissues, they are equipped with multiple protective mechanisms for constraining harmful insults.

The hematopoietic system represents the benchmark for dissecting DNA-protecting mechanisms within a hierarchical context. After irradiation, the hematopoietic compartment responds in a different way, with adult hematopoietic stem cells (HSC) displaying higher radioresistance than their progeny (5). This differential pattern of response preserves tissue homeostasis, thus enabling HSCs to fulfill local and systemic needs. To accomplish

this function, HSCs are maintained in a quiescent state within privileged bone marrow microenvironments, commonly referred to as niches, which protect HSCs from exhausting their replication potential and minimizing the exposure to DNA-damaging metabolic products (6). If on the one hand, quiescence ensures longevity to HSCs; on the other hand, the slow replication kinetics forces them, when required, to adopt the low-fidelity NHEJ. Therefore, this short-term survival strategy is theoretically burdened by potential detrimental effects on genome integrity and could account for post-chemotherapy/radiotherapy hematologic malignancies and age-related blood disorders. It is worth noting that when forced to cycle, HSCs continue to opt for DNA repair instead of cell death (7). Unlike quiescent HSCs, however, cycling hematopoietic precursors take advantage of the error-free HRRs. This observation further highlights the crucial role of cell-cycle kinetics on the fidelity of DNA repair. Finally, HSCs at different ontogenetic stages express a distinct pattern of response upon DNA damage. Highly proliferative umbilical cord blood-derived HSCs display a slower rate of DSBs compared with more differentiated progenitors together with a massive recruitment of apoptotic mediators (8). This indicates that programmed cell death is the main outcome of DNA damage at this developmental stage. This response is consistent with the need to establish a proficient pool maintaining blood homeostasis for the whole lifespan.

Similarly, epidermal stem cells express greater resistance to DNA-damaging agents than to all other cells of the epidermis, as indicated by higher expression of anti-apoptotic molecules, shorter p53 activation, and enhanced NHEJ activity (2). These mechanisms are shared by other tissue-resident stem cells, thus indicating that stem cells have evolved highly efficient repair mechanisms (2). Notwithstanding, quantitative (reduced self-renewal or differentiation potential) or qualitative (mutations) alterations can perturb stem cell homeostasis, leading to the onset of degenerative and neoplastic diseases, respectively.

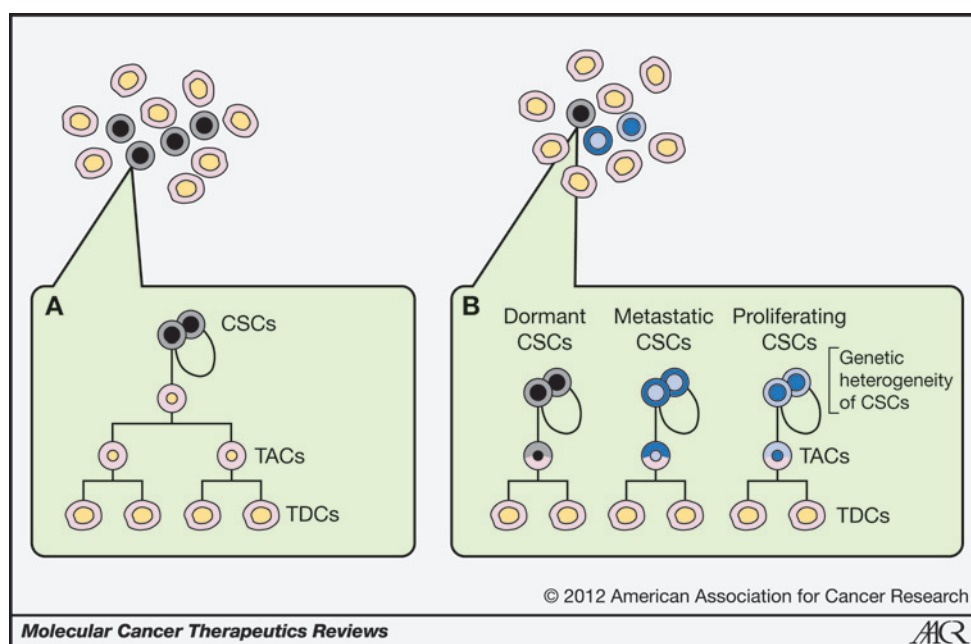
### The Cancer Stem Cell Model

The "cancer stem cell theory" has captured great attention following the identification of a rare population of leukemia-initiating cells possessing stem-like features (9) and has been further strengthened by the isolation and characterization of tumor-initiating, stem-like cells in almost all solid tumors (10–13). According to this model, an uncommon population of tumor cells endowed with self-renewal ability and therefore referred to as cancer stem cells (CSC), accounts for tumor initiation, progression, and treatment failure. This allowed to envision tumors as organized, like normal tissues, in a hierarchical manner (hierarchical model) with a CSC occupying the apex of the pyramid and serving as the precursor of whole tumor population. The logic behind the CSC theory originally fueled an intense debate having questioned the "clonal evolution model." This theory postulates that

mutant clones, each one with the same ability to proliferate and to retain tumorigenicity, cohabit the tumor competing with each other to ensure nutrients and endure to microenvironmental perturbations. However, compelling evidence indicates that only few cancer cells are actually tumorigenic and that these tumorigenic cells could be considered CSCs. To this regard, compared with total bulk cells, CSCs express higher levels of stem cell genes (14, 15), possess clonogenicity *in vitro*, and higher tumorigenic potential *in vivo* (10, 16). Moreover, the unexpected complexity of the tumor hierarchy has been recently confirmed following the isolation of multiple colon cancer-initiating clones with distinct properties upon serial transplantation into the murine background (Fig. 1; ref. 17).

The conclusion drawn by the stem-like phenotype of tumor-initiating cells is that stem cells are the target of the oncogenic process. This has fostered the translation of knowledge about stem cell biology to the pathobiology of cancer, based on the assumption that stem cells undergoing malignant transformation generate cancer cells that retain, although in a distorted manner, their functional properties. The concept that CSCs are reminiscent of their origin is supported by the imbalanced distribution of self-renewal effectors between CSCs and their differentiated progeny (18). This is corroborated by the preferential depletion of CSCs following the pharmacologic abrogation of self-renewal pathway components (19). It is also believed, although not fully proven yet, that the necessary stimuli exploited by CSCs to retain both self-renewal and differentiation ability are the results of their interaction with the environment in which they reside (20). Consistent with this, paracrine-acting, self-renewal-associated pathways have been linked with the epithelial–mesenchymal transition (21), a genetic program inducing both prometastatic traits and stem-like features in cancer cells (22). The gain of "stemness" by neoplastic cells is also elicited by physiologic conditions existing within niches, such as hypoxia and low pH (23, 24). In turn, CSCs thrive also by their ability to recreate optimal microenvironmental conditions, as suggested by their direct differentiation into endothelial-like cells (25, 26).

The relative abundance of CSCs in tumoral tissues has been correlated with both the prognosis of patients with cancer and the efficacy of anticancer treatments. An inverse relationship exists between the *in vitro* growth potential of CSCs and clinical outcomes of patients with glioblastoma treated with standard surgical resection followed by adjuvant chemoradiotherapy (27). Similarly, an increased sphere-forming ability has been reported following neoadjuvant chemotherapy for treating patients with breast cancer (28). This finding suggests greater chemoresistance of CSCs in comparison to the bulk of tumor cells. An optimal discovery validation path of CSC-related signatures might have deep implications for both individual risk assessment and identification of targetable pathways. For instance, an "invasiveness gene signature" composed of 186



**Figure 1.** The evolution of the CSC concept. A, the CSC model originally postulated that a stem-like cell occupying the apex of the tumor pyramid is the precursor of the whole tumor population, and its intrinsic plasticity accounts for the heterogeneity characterizing cancer tissues. B, the apex of the pyramid seems to be more complex, being composed by distinct CSCs, with a distinct genetic background and a different biologic behavior. TAC, transit-amplifying cells; TDC, terminally differentiated cells.

differentially expressed genes in breast CSCs compared with normal breast epithelium has been associated with overall and metastasis-free survival of patients with breast cancer (29). More recently, germ line polymorphisms in colon CSC-related genes have been linked to the time to tumor recurrence in high-risk patients with stage II and stage III colon cancer treated with standard adjuvant chemotherapy (30). However, the relative small cohort of patients examined in these studies and their retrospective nature impose further investigations to assess the prognostic/predictive value of CSC-related parameters in the clinical setting.

### DNA Repair in CSCs

Given the homologies existing between stem cells and their malignant counterpart, it is not surprising that CSCs possess similar defensive mechanisms. The connection between DNA repair signals and CSCs chemoresistance stems from studies carried out in high-grade primary brain tumors. In a pioneering report, CD133<sup>+</sup> glioblastoma stem cells activated ATM and Chk1 more promptly than the CD133<sup>-</sup> counterpart (31). This molecular response enabled CD133<sup>+</sup> cells to survive ionizing radiation, as opposed to the CD133<sup>-</sup> population that underwent cell death. Notably, radiosensitivity was restored by the pharmacologic abrogation of Chk1 and Chk2. Subsequent evidence, however, failed to confirm that the glioblastoma stem cells pool reacts with enhanced DNA repair activity following exposure to ionizing radiation. In particular, 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 (32). 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<sup>+</sup> cells exhibit reduced DSB repair ability (33). Cell-cycle analysis revealed that although glioblastoma stem cells possessed intact G<sub>2</sub> 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. However, preclinical studies continue to be inconsistent. Recently, unsupervised hierarchical clustering analysis of gene expression data, provided by The Cancer Genome Atlas Network, revealed that high-grade primary brain tumors can be grouped in subtypes (proneural, classical, mesenchymal, and neural). Each molecular asset is characterized by a different composition of somatic mutations in master oncogenes and oncosuppressors such as EGF receptor (*EGFR*), *CDKN2A*, *PDGFRA*, *NF1*, *p53*, and *PTEN* (34). Therefore, this genetic heterogeneity could mirror a different DNA damage repair proficiency among subtypes, thus providing a possible explanation for the conflicting results discussed above. Next, 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 (35). 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 (36), thus

shedding doubts on the biologic relevance of this pathway on survival of temozolomide-treated glioblastoma stem cells. High-grade primary brain tumors are also known to aberrantly activate the phosphoinositide 3-kinase (PI3K)/Akt pathway (37), 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 (38). It is worth considering that the pharmacologic abrogation of Akt impaired glioblastoma stem cells fitness and abrogated neurosphere formation (39), thus allowing to postulate that Akt inhibitors could be exploited as chemotherapy-enhancing agents. Although the mechanistic link between checkpoint-associated molecules and glioblastoma stem cells survival upon genotoxic injuries is still debated, similar survival mechanisms are adopted by other CSC types. For instance, significant increases in the expression of DNA repair- and cell-cycle-related genes have been observed in pancreatic CSCs compared with bulk cells after challenge with gemcitabine (40). Using OncoPrint database coupled with Ingenuity Pathways Analysis (IPA), a significant increase in DNA copy number of BRCA1 and RAD51 has been observed in prostatic CSCs compared with adherent population isolated from the primary site (41). Moreover, both colon and lung CSCs, unlike their differentiated progeny, efficiently activate Chk1 when exposed to standard chemotherapeutic agents (42, 43). While the aberrant activation of G<sub>2</sub>-M checkpoint controllers conferred chemoresistance, their pharmacologic inhibition significantly increased chemosensitivity by triggering a modality of cell death, known as mitotic catastrophe, aimed at eliminating mitosis-incompetent cells. These data indicate that a proficient DNA repair mechanism exploited by CSCs may be responsible for the partial inefficiency of current treatments and urge the need for a well-designed CSC-tailored therapy.

Whether brain tumor models have been widely used for studying DNA repair pathways at the preclinical level, breast cancers harboring germ line mutations in the HRR-associated proteins BRCA1 and BRCA2 are the benchmark for proof-of-principle clinical trials aimed at assessing the antitumor activity of DNA repair pathway inhibitors. Enhanced DNA repair ability has been also described in breast CSCs whose isolation and characterization provided the first hint supporting the CSC model in solid tumors (10). Transcriptional profiling of the putative CSC population isolated from the mammary gland of p53-null mice indicated that these cells were enriched in both DNA repair- and self-renewal-linked genes (44). Array-based gene expression analysis conducted by the Affymetrix HuGene 1.0 ST Array, conducted in our laboratory, revealed that breast CSCs, isolated from primary xenograft-derived tumors, and metastatic derivatives exhibit higher levels of DNA repair-linked effectors such as BRCA1, ATR, ATM, and Chk1 when compared with differentiated tumor cells (Bartucci and colleagues, unpublished results). Furthermore, mammospheres from the commercial cell line MCF-7 displayed a more active

DNA single-strand break repair (SSBR) pathway in comparison to the bulk population, as indicated by higher levels of the SSBR-associated protein APE1 (45). Moreover, long-term exposure of MCF-7/ADR cells to doxorubicin led to gaining stem-like properties coupled with enhanced chemoresistance-conferring mechanisms (46), as documented by the increased expression of genes encoding multidrug resistance-related proteins and the cyclophosphamide-metabolizing enzyme aldehyde dehydrogenase 1. Radioresistance of breast CSCs seems to be also sustained by lower concentrations of ROS (47). This phenomenon is due to an increased expression of free radical scavenger systems, such as those belonging to the glutathione metabolism, which counteracts the effects of water radiolysis, the main modality of ionizing radiation-induced cell death. This radioresistant phenotype was reverted by the inhibition of glutathione metabolism, which restored breast CSC radiosensitivity and decreased their clonogenic potential. To further enforce the interconnection between stemness-associated pathways and DNA repair signals, it has been reported that the aberrant activation of both the canonical WNT pathway and Akt conferred radioresistance to breast CSCs, whereas Akt neutralization sensitized breast CSCs to radiotherapy via the inhibition of  $\beta$ -catenin (48). Although the role of DNA repair pathways in determining chemoradioresistance of breast CSCs seems to be less contradictory than in brain tumors, data should be interpreted with caution. In fact, breast cancer is a constellation of molecular and clinical entities, each one characterized by a distinct clinical behavior, a different molecular portrait, and a different degree of responsiveness to chemotherapy, hormone therapy, and molecular-targeted agents. Therefore, the molecular taxonomy of breast cancer implies that optimal development of DNA repair inhibitors should be carried out within well-defined genetic contexts.

It is known that stem cell longevity is ensured by prolonged exit from the cell cycle, a mechanism that prevents the exhaustion of the replicative potential and limits DNA damage (49). Experimental evidence indicates that both *in vitro* and *in vivo*, a subpopulation of slow-cycling tumor cells is mostly spared by chemotherapy-induced death when compared with the bulk of the cells (50, 51). Ovarian and pancreatic cancer label-retaining population, both encompassing the operative criteria to be defined as CSCs, were able to survive, unlike non-label-retaining cells, standard chemotherapeutic agents (50, 51). The existence of quiescent CSCs has fostered the development of pharmacologic strategies able to target "dormant" cells, and some compounds including cytokines (IFN- $\alpha$  and granulocyte colony-stimulating factor) or chemicals (arsenic trioxide) seem to be endowed with such properties (52). The list of potential "quiescence-disrupting" agents has been further implemented with epigenetic-acting histone deacetylase inhibitors (HDACi). The first-in-class HDACi vorinostat, a compound approved for treating refractory cutaneous T-cell lymphoma, successfully induced apoptosis in quiescent

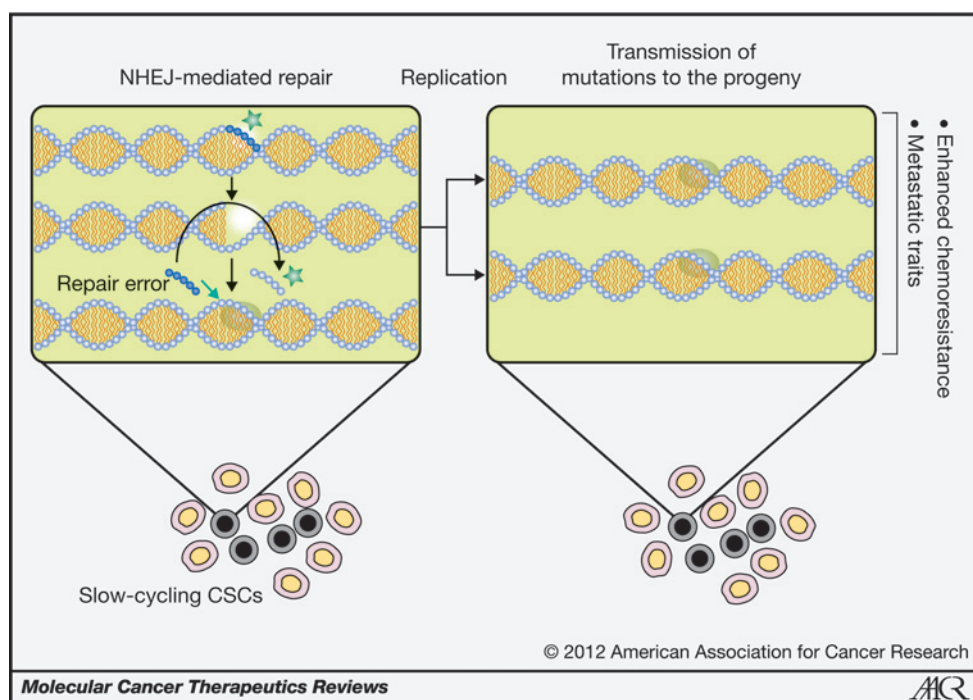


Figure 2. The relationship between cell cycle and DNA repair fidelity. When damaged, quiescent/slowly cycling CSCs are forced to use the error-prone NHEJ pathway. This repair strategy could paradoxically generate a new mutation that is passed down to the progeny. The resulting offspring can therefore display enhanced malignant properties such as metastatic ability or increased chemoresistance.

chronic myelogenous leukemia stem cells when combined with imatinib mesylate (53). However, given the tight relationship existing between DNA repair strategies and cell cycle, CSCs are probably forced to use the error-prone NHEJ (Fig. 2). Therefore, quiescence potentially contributes to both chemoresistance and genetic instability of CSCs, whereas co-targeting quiescence-associated molecules and DNA repair pathway effectors might contribute to an efficient eradication of CSCs.

### DNA Repair Pathway Inhibitors and Companion Biomarkers

Early clinical trials aimed at assessing the activity of DNA repair inhibitors have been carried out with the MGMT-depleting agents *O*<sub>6</sub>-benzylguanine and lomeguatrib in combination with carmustine and temozolomide, respectively (54, 55). Although an efficient depletion of the target was documented in the pharmacodynamic assay, results were disappointing; perhaps, the unacceptable toxicity observed with standard schedules was required to adopt suboptimal chemotherapy doses.

More recently, a second wave of clinical trials with chemotherapy-enhancing therapeutic approaches has been conducted for determining the antitumor activity of PARP inhibitors. Currently, at least 9 of these molecules are in clinical or late preclinical development. PARPs are nuclear enzymes with multiple functions and, among components of the family, PARP-1 and PARP-2 are involved in single-strand repair via the BER pathway. The molecular background underlying the development of PARP inhibitors is a modality of

gene-gene interaction known as synthetic lethality. According to this model, while a mutation confers an advantage for cancer cells, the concomitant pharmacologic abrogation of a redundant pathway significantly affects cell fitness. Because BRCA-deficient cells have impaired HRRs, they are forced to use the BER pathway for repairing persistent SSBs. As a result, PARP neutralization makes BRCA-deficient cells unable to use this alternative pathway when exposed to DNA-damaging agents, thus leading to cell death. This approach has been exploited for targeting breast and ovarian cancers carrying *BRCA1* or *BRCA2* germ line mutations. A phase II multicenter study conducted in patients with advanced, refractory breast cancer whose tumors harbored *BRCA* mutations evaluated 2 different doses of olaparib (AZD2281) in 2 sequential cohorts (27 + 27 patients; ref. 56). The overall response rate was 41% (11 patients) and 22% (6 patients) with olaparib given at 400 mg and 100 mg, respectively, with an acceptable safety profile. Such promising, although initial, results were confirmed in 57 *BRCA*-mutated carriers with ovarian cancer (57). More recently, a phase II, open-label, nonrandomized study conducted in patients with high-grade serous and/or undifferentiated ovarian cancer and advanced triple-negative breast cancer confirmed positive results against ovarian cancer carrying *BRCA1* or *BRCA2* mutations. Although to a lower extent, the antitumor efficacy was also documented in the nonmutant background (58). However, no confirmed objective responses were reported in patients with breast cancer. Iniparib (BSI-201), another PARP inhibitor, has been recently evaluated in a randomized, open-label, phase II study trial in combination with

carboplatin/gemcitabine for treating metastatic triple-negative breast cancers (59). Consistent with the fact that this breast cancer subtype displays dysregulation of *BRCA1* and, therefore, shares molecular and clinical features with hereditary *BRCA1*-related breast cancers (BRCAness phenotype; ref. 60), adding iniparib to alkylating agent-based chemotherapy significantly improved both clinical benefit and overall response rate. The clinical benefit rate was 56% in the experimental arm compared with 34% in the chemotherapy-alone arm. The overall response rate also favored the investigational treatment (32% vs. 52%). All other endpoints (median progression-free survival and median overall survival) were improved with the iniparib-containing regimen, although the trial was not powered to look at long-term outcomes. However, the subsequent phase III trial unexpectedly failed to improve overall and progression-free survival, and molecular analysis is underway in the attempt of identifying the subset of responder patients (61). A possible explanation for such unsatisfying results is that iniparib, as opposed to others PARP inhibitors, mainly acts by inducing cell-cycle arrest in G<sub>2</sub>-M phase rather than inhibiting PARP-1 and PARP-2, at least at physiologic drug concentrations.

While current molecular-targeted agents are directed against proto-oncogenic proteins, lethal interaction-based therapy is offering the opportunity for targeting, although indirectly, deregulated oncosuppressors. This rationale has been also proposed for developing Chk1 inhibitors. Because p53-defective cells are unable to undergo G<sub>1</sub> arrest, they depend on alternative checkpoint activators to arrest the cell cycle in response to DNA damages. Conversely, cells with intact p53-dependent checkpoint are expected to be unperturbed, thus implying that normal cells should be spared from the sensitization to DNA-damaging agents. Many Chk1 inhibitors showed chemosensitizing properties in the preclinical setting and are undergoing early phases of clinical development (AZD7762, PF-477736, SCH900776, LY2606368; ref. 3). Notwithstanding, 2 main concerns recently arose from preclinical evidence and early clinical data. The preferential antitumor activity of Chk1 inhibitors against p53-defective cells has been questioned. In particular, both short-term cell survival and long-term colony-forming ability have been reported to be independent from p53 status following Chk1 neutralization (62). This finding brings into question the predictive value of p53 status and, therefore, could negatively impact the biomarker-driven development of Chk1 inhibitors. Second, although Chk1 antagonists were thought to have a favorable therapeutic index, two phase I dose-escalation trials with AZD7762 in combination with either gemcitabine or irinotecan reported an unexpected cardiotoxicity, which led to withdrawing this compound from the market (63, 64). Because cardiac dose-limiting toxicity was also observed with the Chk1 inhibitor SCH900776 in combination with gemcitabine (65), and considering that the above-mentioned chemotherapeutic agents are not associated with an increased

risk of cardiac events, whether cardiotoxicity is a class effect of Chk1 antagonists needs to be urgently addressed. Agents targeting G<sub>2</sub> phase checkpoint members acting downstream of Chk1, such as Wee1, have been developed with the same conceptual approach proposed for Chk1 inhibitors. The Wee1 inhibitor MK-1775 potentiates the activity of many DNA-damaging agents such as platinum derivatives, both *in vitro* and *in vivo*, mainly in a p53-dependent manner (66). Although MK-1775 enhanced radiosensitivity in commercial glioblastoma cell lines without affecting normal human astrocytes, similar properties have not been confirmed against glioblastoma stem cells (67).

Such an expanding pipeline of DNA damage-interfering agents has fostered the identification of pharmacodynamic biomarkers coupled with robust and reliable tests for detecting DNA damage-related endpoints. DSB-induced  $\gamma$ -H2AX foci are widely used as a biodosimeter for measuring the extent of genotoxic injuries induced by the exposure to chemicals or radiation. In recent years, this parameter has moved from the laboratory to be used in early clinical trials with DNA damage repair antagonists (68). One of the most challenging aspects in the early development of molecular-targeted agents is the need for multiple biologic samples for monitoring the target over time. To overcome this drawback,  $\gamma$ -H2AX levels have been evaluated in health tissues (circulating blood cells, buccal cells, hairs) as a surrogate parameter of drug-induced DNA damage. However, the exploitation of surrogate tissues instead of the tumor for measuring biologic outcomes has some intrinsic limitations, correlated with both the cell type evaluated and its replicative state. To this end, the determination of  $\gamma$ -H2AX on circulating tumor cells will enable investigators to conduct multiple measurements in the target cells in a minimally invasive way (69). Finally, a high-throughput screening system (RABIT-Rapid Automated Biodosimetry Tool), based on an established  $\gamma$ -H2AX immunofluorescence assay, has been developed to screen thousands of samples per day (70). When considering DNA repair-linked biomarkers, however, the attention turns to the enzyme repair cross-complementation group 1 (ERCC1), an NER component whose levels have been traditionally associated with the benefit of patients with non-small cell lung cancer (NSCLC) platinum-containing doublets. Although the predictive value of this biomarker remains to be addressed, a recent meta-analysis suggested that low ERCC1 levels are associated with a higher objective response and longer survival in patients with advanced NSCLCs (71). In addition, the molecular analysis of 1,207 NSCLCs revealed a strong association between low ERCC1 mRNA levels and EGFR-activating mutations (72). This observation implies that the co-detection of such molecular determinants could identify a subset of NSCLCs with a marked sensitivity to both EGFR-tyrosine kinase inhibitors and platinum-containing chemotherapy. The

connection between DNA repair ability and outcomes of patients with NSCLCs receiving platinum-based chemotherapy has been further enforced by the existence of an inverse relationship between DNA repair capacity, measured *in vitro* in lymphocytes with the host cell reactivation assay, and patients survival (73). Finally, it is known that approximately 15% of sporadic colorectal cancers are characterized by microsatellite instability (MSI; ref. 74). This form of genetic instability is sustained by alterations in MMR components, consisting of either germ line mutations in one of the MMR genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) or epigenetic silencing of *MLH1*. This colorectal cancer subtype presents distinct clinicopathologic features such as right-sided location, lymphocytic infiltration, mucinous histology, and poor differentiation. The MSI phenotype has been associated with better prognosis and reduced likelihood of metastasis compared with microsatellite-stable tumors, even though patients whose tumors harbor the MSI phenotype have a less pronounced benefit from 5-fluorouracil-based chemotherapy

## Conclusions

The exact definition of the target population is a crucial element for optimal preclinical development of molecular-targeted agents. Growing evidence points to CSC eradication as the most valuable strategy for achieving long-lasting tumor remission. However, CSCs are protected against standard medical treatments by multiple mechanisms, also including the abnormal activation of DNA damage repair signals. We believe that 3 main questions need to be addressed to fully dissect DNA repair pathways as therapeutic targets within the pyramidal organization of tumors. First, commercial cancer cell lines have been traditionally used for generating tumors in mice, although these cells are unable to give rise to a tumor resembling the human disease. Given the possibility to expand *in vitro* CSCs, the generation of CSC-based tumor xenografts able to recapitulate the parental tumor into the murine background is now considered as the gold standard for evaluating the anticancer properties of experimental agents. This opportunity has been welcomed as a major advance in experimental oncology. The genetic heterogeneity existing at the apex of the tumor pyramid should be however considered. The distinct biologic behavior of tumor-initiating subpopulations is indeed the potential source of discrepant results, even within the same experimental model, coming from studies aimed at dissecting the role of DNA damage pathways in CSC chemoradioresistance. Second, a rationale development of DNA repair inhibitors and, more in general, of molecular-targeted agents should be ideally carried out within a defined genetic background. This stems from evidence showing that the majority of biomolecules used in the clinical setting are effective in the presence of specific genetic alterations. When consider-

ing DNA repair inhibitors, 2 different, but complementary, approaches could be exploited by taking advantage of high-throughput RNA interference screening assays. These tools make it possible to identify molecular networks whose abrogation produces the expected outcome only in the presence of a predefined molecular parameter (mutation) and therefore to determine the extent to which DNA repair components are represented in such circuits. Similarly, mutant effectors of the DNA repair machinery could be adopted as the reference parameter, thus allowing the identification of pharmacologic strategies for targeting cells carrying genetic defects in DNA damage pathways. The logic behind this approach is the co-development of DNA damage inhibitors and companion biomarkers, a procedure that will enable physicians to plan clinical trials in selected patient populations. It is plausible that this approach will significantly shorten the developmental path of new agents that can be evaluated, for instance, in multiarm trials with an adaptive randomization design while avoiding the need for large randomized phase III trials in unselected patients. Third, chemotherapy-enhancing agents aimed at eliminating CSCs could be burdened by severe side effects due to "off-target" effects on normal stem cells. Although molecular-targeted agents have been traditionally considered to be safe, this paradigm is rapidly changing. Safety concerns have halted clinical trials with promising biomolecules. Neratinib, for instance, is an irreversible pan-ErbB receptor tyrosine kinase inhibitor whose antitumor activity was limited by diarrhea-related dose reduction (75). It is evident that a deeper characterization of mechanisms protecting stem cells is needed, optimally requiring a panel of human stem cells representing the most critical tissues as "safety control" during preclinical development of potential anti-CSCs drugs. We believe that this is crucial to avoid clinical trials to stop early and/or dose reduction of the chemotherapy backbone.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** M. Maugeri-Saccà, M. Bartucci, R. De Maria  
**Writing, review, and/or revision of the manuscript:** M. Maugeri-Saccà, M. Bartucci  
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