Chapter 2
Nitric Oxide and Genomic Stability

Vasily A. Yakovlev

Abstract Epidemiological evidence accumulating over the years has provided a positive correlation between cancer incidence and chronic inflammation. Regardless of etiology, inflammatory conditions are characterized by overexpression of inducible nitric oxide synthase (iNOS) and overproduction of nitric oxide/reactive nitrogen species (NO/RNS) in epithelial and inflammatory cells at the site of carcinogenesis. NO/RNS produced in infected and inflamed tissues can contribute to the process of carcinogenesis by different mechanisms. In this chapter, we discuss NO/RNS-dependent mechanisms of genomic instability (GI) and bystander effects. We explain the mechanism of "synthetic lethality" of the NO-donor/PARP-inhibitor combination and its role in sensitization of the cancer cells to DNA-damaging agents. We postulate the "mutator field" theory and the definition of mutagenesis efficacy.

Keywords BRCA1 · Bystander effect · Chronic inflammation · Genomic instability · Homologous recombination · Nitric Oxide · Non-homologous end joining · NOS · RAD51 · Synthetic lethality

Abbreviations

BDD	Bystander DNA damage
BER	Base excision repair
BRCA1	Breast cancer type 1 susceptibility protein;
DSB	Double-strand brake
EADC	Esophageal adenocarcinoma
eNOS	Endothelial nitric oxide synthase
GERD	Gastro-esophageal reflux disease
HCC	Hepatocellular carcinoma

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HRND	High concentration range of NO-donors
IBDs	Inflammatory bowel diseases
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharides
MN	Micronuclei
MRND	Moderate concentration range of NO-donors
NHEJ	Non-homologous end joining
NHL	Non-Hodgkin lymphoma
NO/RNS	Nitric oxide/reactive nitrogen species
OLP	Oral lichen planus
OSCC	Oral squamous cell carcinoma
OV	Opisthorchis viverrini
PARP1	Poly(ADP-ribose) polymerase 1
PC	Prostate cancer
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphisms
SSB	Single-strand breaks
SSF	Stress signal factors

Inflammation and Carcinogenesis: Role of NO/RNS Generation

The link between inflammation and cancer was proposed more than 150 years ago when Virchow suggested that malignancies tend to arise at sites of chronic inflammation. Epidemiological evidence accumulating over the years has provided a positive correlation between cancer incidence and chronic inflammation [2], and it is now a well-recognized hallmark of cancer development [3–5]. Infection is proposed to contribute to carcinogenesis through inflammation- related mechanisms. Infection with hepatitis C virus, *Helicobacter pylori* and the liver fluke, Opisthorchis viverrini (OV), are important risk factors for hepatocellular carcinoma (HCC), gastric cancer, and cholangiocarcinoma, respectively. Inflammatory bowel diseases (IBDs) and oral diseases, such as oral lichen planus (OLP) and leukoplakia, are associated with colon carcinogenesis and oral squamous cell carcinoma (OSCC). Chronic gastro-esophageal reflux disease (GERD) underlies molecular progression to esophageal adenocarcinoma (EADC). Regardless of etiology, all these conditions are characterized by overexpression of inducible nitric oxide synthase (iNOS), overproduction of NO/RNS in epithelial and inflammatory cells at the site of carcinogenesis, and formation of DNA damages [6–16]. Antibacterial, antiviral, and antiparasitic drugs dramatically diminished the inflammatory stimulated iNOS expression and formation of the DNA lesions. iNOS inhibitors and NO/RNS-scavengers also significantly reduced DNA damages [13, 17, 18]. Therefore, it is considered that excessive amounts of reactive nitrogen species produced via iNOS during chronic

inflammation may play a key role in stimulation of DNA damages formation and activation of carcinogenesis.

Non-Inflammatory Stimulation of NO/RNS Generation and Its Role in Carcinogenesis

Not only inflammatory conditions can stimulate different types of NOSs. It would be logical to assume that any prolonged stimulation of NO/RNS production can lead to accumulation of DNA lesions and carcinogenesis. For example, the activity of NOSs can be stimulated by single nucleotide polymorphisms (SNPs). It was shown that SNP of the iNOS gene (*NOS2A* Ser608Leu) was responsible for a twofold risk increase for non-Hodgkin lymphoma (NHL) (OR = 2.2, 95 % CI = 1.1–4.4). This risk increase was consistent by cell lineage (B- and T-cell NHL) and pronounced for the two most common subtypes, diffuse large B-cell (OR = 3.4,95 % CI = 1.5–7.8) and follicular lymphomas (OR = 2.6, 95 % CI = 1.0-6.8) [19]. Another group of investigators revealed that the polymorphism in the promoter region of the *endothelial NOS (eNOS)* gene (786Thr > Cys) was the most important promoter alteration of the *eNOS* gene, which significanlty affected the prostate cancer (PC) progression. The incorporation of the Cys allele was associated with increased levels of eNOS transcripts and responsible for variations in the plasma NO, which may promote cancer progression by providing a selective growth advantage to a tumor. The authors suggested that NOS3 transcript level may be used as a biomarker together with the PCA3 marker for molecular staging of the PC [20].

NO/RNS: Different Concentrations, Different Effects

NO/RNS production is often associated with contradictory effects on cell proliferation and cytotoxicity, variably promoting and inhibiting apoptosis in normal and tumor cells [21–23]. Wink and coworkers have examined these contradictory observations and have proposed a set of five graduated levels of NO/RNS cellular responses that range from the promotion of cell survival and proliferation at low concentrations of NO/RNS to the promotion of cell cycle arrest and apoptosis at high concentrations of NO/RNS [21]. While high concentrations of NO/RNS can cause direct DNA damage and stimulate DNA double-strand brake (DSB), there is an emerging appreciation for determining the role of lower NO/RNS concentrations in signaling pathways related to apoptosis, cell cycle, and other facets of cell functions.

Previous reports show that NO/RNS production correlates with NO donor concentrations and time of incubation [24]. Measurements, using an NO-specific electrode, of actual NO concentrations during cell exposure to 125 to 500 μmol/L

of DETA indicated relatively constant NO concentrations in the 150–400 nmol/L range [24–26]. The NO concentrations produced *in vivo* at sites of colonic crypt chronic inflammation and airway inflammation were below 300 nmol/L and below 400 nmol/L, respectively [25, 27]. Many solid tumors grow in the inflammatory microenvironment [28–30], and it was demonstrated that the NO concentration was significantly higher than in the normal tissue. The NO concentration in the cutaneous melanoma reaches 200 nmol/L with the maximum on the periphery of the tumor [31]. In comparison, normal *in vivo* NO concentrations in the absence of inflammation are unlikely to exceed 50 nmol/L [8]. Hence, the amount of NO produced from ~ 100 to ~ 350 μmol/L of NO-donors (DETA NONOate or SNAP) *in vitro* represents the NO concentrations maintained *in vivo* at sites of the chronic inflammation and the growing edge of melanoma. This moderate concentration range of NO-donors (MRND) has a very interesting number of qualities (Fig. 2.1): A. It doesn't stimulate direct DNA damage and, as a result, doesn't affect ATM/ATR-dependent pathways [32]; B. It doesn't inhibit cell proliferation (in fact, 50–200 μmol/L of DETA NONOate or SNAP demonstrates a stimulatory effect on proliferation of the different cell lines); C. It significantly down-regulates error-free homologous

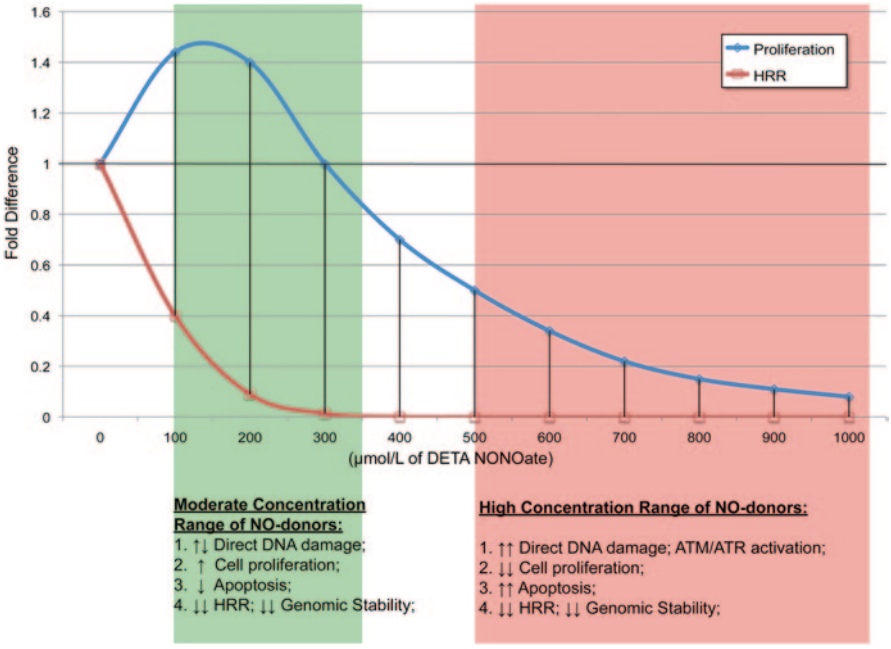

Fig. 2.1 Schematic representation of changes in the cell proliferation and DNA Homologous Recombination Repair (HRR) as a function of NO-donor (DETA NONOate) concentration. The *green zone* represents moderate concentration range of NO-donors in vitro and corresponds to NO/RNS concentrations under chronic inflammatory conditions and tumors inflammatory microenvironment in vivo. The *red zone* represents high concentration range of NO-donors, which is widely used for investigation of NO-dependent DNA damage

Table 2.1 The relative fold decrease of HRR by different concentrations of the NO-donors. A-549 (human lung adenocarcinoma epithelial cell line) and MCF-10A (immortalized human breast epithelial cell line) were incubated 18 h with indicated concentrations of NO-donors (SNAP or DETA NONOate). (HRR was measured by DRGFP reporter based assay [1])

NO-donor concentration (μM)	A549		MCF-10 A	
	SNAP	DETA	SNAP	DETA
0	1	1	1	1
50	1.84	1.39	1.54	2.06
100	2.29	2.73	1.95	3.36
200	5.08	20.89	7.4	24.67
300	37.6	188	74	74

recombination repair (HRR) of DNA DSBs (see Table 2.1) and substitutes it by error-prone non-homologous end joining (NHEJ), which stimulates the effect of genomic instability (NO/RNS-dependent mechanisms of HRR-NHEJ substitution will be discussed below). DSBs are serious genetic lesions that must be repaired to prevent catastrophic loss of chromosomes. In general, two classes of mechanisms exist for repairing DSBs, HRR or NHEJ [33]. HRR requires an identical (or nearly identical) template strand of DNA to mend a lesion whereas NHEJ repairs a double-strand gap in DNA without a homologous template. NHEJ is guided entirely by information in the lesion, which makes NHEJ error prone in comparison to HRR.

As it was shown by different groups of investigators, significant DNA damage as well as stimulation of DNA-damage signaling pathways (for example: ATM/ATR-dependent phosphorylation and activation of p53) can be achieved by using a high concentration range of NO-donors (HRND) > 500 μmol/L of NO-donor [34–36, 32]. The HRND is also characterized by significant inhibition of the cell proliferation and the activation of apoptosis (Fig. 2.1).

If we compare the efficacy of MRND and HRND with respect to mutagenesis, MRND allows the generation of DNA errors by switching from error free HRR to error-prone NHEJ. Also, stimulation of cell proliferation and downregulation of apoptosis by MRND of NO-donors facilitate the accumulation of the DNA errors in new generations of cells. On the other hand, HRND generates much more DNA errors by downregulation of HRR and by direct DNA damage. However, inhibition of the cell proliferation and the activation of apoptosis leads to elimination of most of the affected cells and prevents the accumulation of DNA mutations in the next cells generations. Hence, MRND, as well as the NO/RNS concentrations maintained *in vivo* at sites of chronic inflammation and growing edges of tumors, can be characterized as the ***most favorable environment for mutagenesis*** (MFEM) (see Fig. 2.1).

Mechanisms of NO/RNS-Dependent Mutagenesis

NO/RNS produced in infected and inflamed tissues can contribute to the process of carcinogenesis by different mechanisms. NO/RNS mediate cellular regulation through the posttranslational modifications of a number of regulatory proteins. The best studied of these modifications are S-nitrosylation (reversible oxidation of cysteine) [37–39] and tyrosine nitration [40–42]. NO/RNS-dependent posttranslational proteins modifications (Tyrosine nitration and S-nitrosylation) are well-accepted markers of the tissue inflammation, also stimulating attention by their significant impact to carcinogenesis and tumor growth. These modifications can up- or down-regulate functions of many proteins. The breast cancer type 1 susceptibility protein (BRCA1) contributes to cell viability in multiple ways, it plays a critical role in HRR of DNA DSBs, cell cycle checkpoint control, transcription, and regulation of chromosome segregation [43–45]. The loss of BRCA1 protein function predisposes to the development of breast and ovarian cancers [46].

BRCA1 expression is negatively regulated at the transcriptional level by the repressive complex of retinoblastoma-like protein 2 (RBL2) and E2F4. Formation of the repression RBL2/E2F4 complex can be accelerated by, for example, RBL2 dephosphorylation. Recently, protein phosphatase 2 A (PP2A), an enzyme responsible for RBL2 dephosphorylation, was shown to be activated by nitration on Tyr284 [47]. Inflammatory levels of NO/RNS, which don't induce significant DNA damage and maintains the ATM/ATR-dependent pathways intact, stimulate substantial dephosphorylation of RBL2. RBL2 dephosphorylation promotes a repressive RBL2/E2F4 complex formation with subsequent block of BRCA1 expression (Fig. 2.2) [1]. As result, BRCA1-dependent mechanisms of genomic stability can be significantly compromised. Interestingly, the same mechanism of BRCA1 downregulation takes place in the different types of cells under hypoxic condition [48]. That NOS activity and NO/RNS generations are stimulated under certain hypoxic conditions [49–51] suggest that some pro-carcinogenic effects of hypoxic microenvironment are also NO/RNS- dependent.

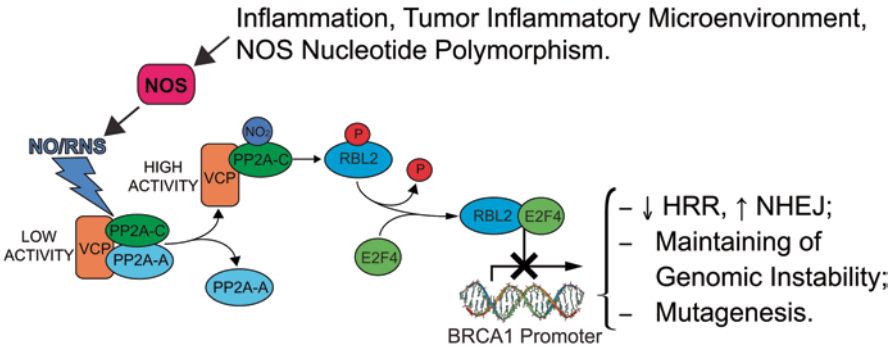

Fig. 2.2 Schematic representation of NO/RNS-dependent inhibition of BRCA1 protein expression

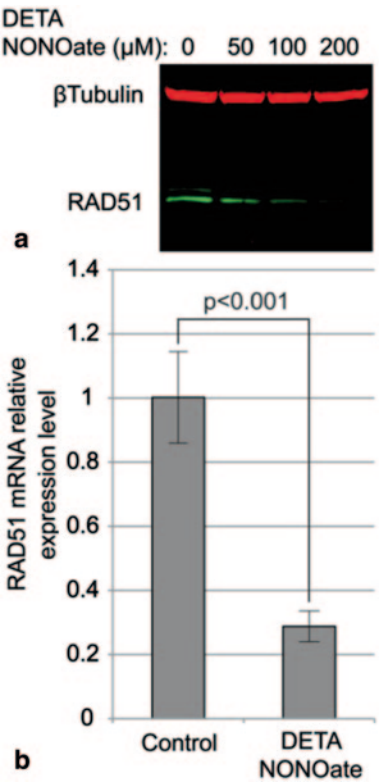

Fig. 2.3 NO/RNS-dependent down regulation of RAD51 protein expression in A549 cell line. A, Level of RAD51 protein after 18h of incubation with different doses of DETA NONOate. B, qPCR analysis of endogenous RAD51 expression after 18h of incubation with 200 μM of DETA NONOate. RAD51 expression was normalized to 18 S mRNA expression. Data are presented as the mean ± SD of 4 experiments. The P value was calculated with the Student t test. (unpublished data)

We recently found that protein RAD51, another critical mediator of HRR, is downregulated by NO/RNS (Fig. 2.3). Analyses of the RAD51 gene promoter activity and mRNA stability suggested that the NO/RNS-dependent regulation of this gene occurs via transcriptional repression (unpublished data). The E2F site in the *RAD51* promoter matches an identical sequence at a similar relative location within the *BRCA1* proximal promoter [52], and this *BRCA1* promoter element also mediates the repression of this gene in response to NO/RNS increase (Fig. 2.2). Hence, the expression of both proteins critical for HRR can be affected through the same signaling pathway: RNS/PP2A/RBL2/E2F4/proximal promoter of *BRCA1* and *RAD51*.

Synthetic Lethality: Combination of NO-Donors and PARP-Inhibitors

Poly(ADP-ribose) polymerase 1 (PARP1) is an abundant nuclear enzyme that synthesizes poly(ADP-ribose) polymer when activated by DNA nicks or breaks. Binding of PARP1 to DNA strand breaks is critical for resealing of the DNA sin-

gle-strand breaks (SSB) during base excision repair (BER) [53–57]. Loss of PARP activity results in accumulation of DNA SSBs, which are subsequently converted to DNA DSBs by the cellular replication and/or transcription machinery (see Fig. 2.4). In BRCA-positive cells, these DSBs are repaired by HRR, but they cannot be properly repaired in BRCA-deficient cells, leading to genomic instability, chromosomal rearrangements, and as a result—cell death [58, 59]. This effect is known as a synthetic lethality: when inhibition in either of the two signaling pathways (by gene mutation or chemical inhibitor) is present and remains independently viable, but when present together, the combination results in non-viability.

The role of PARP1 in the DNA damage response promoted the development of PARP inhibitors as chemo- and radio-sensitizers for the treatment of cancers harboring mutations in the *BRCA* genes [60]. Overall, it has been estimated that inherited *BRCA1* or *BRCA2* mutations account for 5–10 % of breast cancers and 10–15 % of ovarian cancers among white women in the United States [61]. However, a low frequency of *BRCA1* loss in non-hereditary tumors can limit the clinical use of this approach [58]. The possibility of applying the synthetic lethality in many types of cancers by lowering BRCA1 expression by pharmacological NO-donor treatments is very appealing (Fig. 2.4). In our recent work, we tested the effect of NO-donor/PARP-inhibitor combination on the sensitization of cancer cell lines to ionizing radiation (IR) (Fig. 2.5).

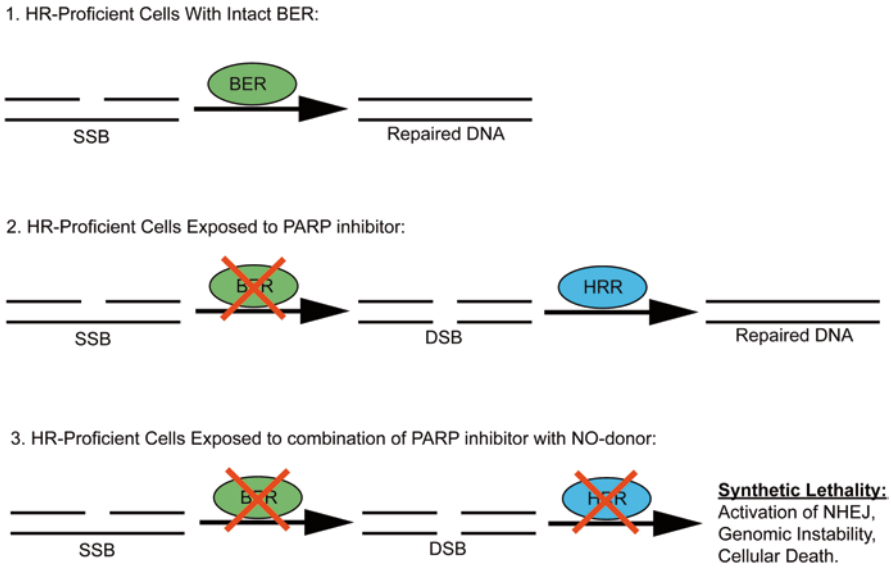

Fig. 2.4 A proposed stimulation of synthetic lethality by combination of PARP- inhibitor and NO-donor

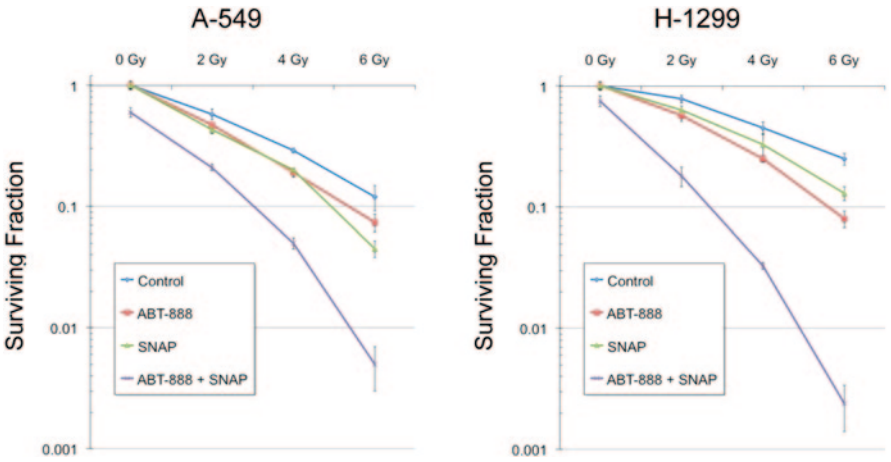

Fig. 2.5 Combined treatment with NO-donor SNAP (200 μM) and PARP inhibitor ABT-888 (10 μM) significantly increased sensitivity of A-549 and H-1299 (human non-small lung carcinoma cell line) to ionizing radiation. (unpublished data)

From Bystander Effect to Mutator Field

The radiation-induced bystander effect (RIBE) was studied widely in the past decades since the description of this phenomenon by Nagasawa and Little in 1992 [25]. It was shown that the irradiated cells release some "stress signal factors" (SSF) to affect the adjacent cells, or to affect the cells, which have received the medium, conditioned by the irradiated cells. These cells, which are not irradiated but are affected by the SSF, are called bystander cells. These SSF cause excess DNA damage, expression of DNA damage related proteins, chromosome aberration, mutations, and malignant transformation in the non- irradiated bystander cells [26].

The uniqueness of nitric oxide (NO•) as a redox signaling molecule resides in part in its relative stability and hydrophobic properties that permit its diffusion through the cytoplasm and plasma membranes over several cell diameter distances [62]. Hence, stimulation of NO• generation can affect different pathways within the cell in which it is produced or penetrates cell membranes to modulate signaling pathways in the bystander cells [63]. Recent studies have demonstrated the important role of NO• in mediating the bystander response induced by low-dose irradiation. Shao et al. [27, 31] presented evidence for a significant increase in the incidence of micronuclei (MN) in the non-irradiated bystander cells that were in the vicinity of ones irradiated through either the nuclei or the cytoplasm with a microbeam facility. Pre-treatment with a scavenger of NO• (c-PTIO) abolished excess of MN formation. Using the DAF-FM diacetate, NO-activated fluorescent probe, and targeted with a precision microbeam, Shao et al. [27] detected an increase in the number of fluorescent-positive cells than the actual number of directly irradiated cells. In another study, Han et al. revealed that the stimulated cell proliferation and the increased MN and DSB were observed simultaneously in the bystander cell

population, which were co-cultured with cells irradiated by low-dose α-particles (1–10 cGy) in a mixed system [64]. NO• played an essential role in the stimulation of these effects in the bystander cell population. Low concentrations of NO•, generated by spermidine (NO-donor), were proved to induce cell proliferation, DSB, and MN simultaneously.

Different factors can stimulate NO• production in target cells and increased DNA damage in bystander ones. Irradiation, as well as, stimulation of RAW 264.7 (mouse leukaemic monocyte macrophage cell line) by LPS induced an iNOS activity and NO• generation, which increased the DNA damage in bystander cells [65]. Pretreatment of target macrophages or bystander cells with the direct NOS inhibitor (L-NAME) significantly reduced the induction of gene expression and DNA damage in the bystander cells.

How NO• can stimulate DNA damage in the bystander cells? Some authors hypothesized that moderate concentrations of NO/RNS stimulate proliferation and shortening of the cell cycle in bystander cells, which gave them insufficient time to repair DSBs. The increased cell division might increase the probability of carcinogenesis in bystander cells since cell proliferation increased the probability of mutation from the mis-repaired DSBs [64]. However, another group of researchers demonstrated that accumulation of bystander DNA damages (BDD) is not dependent on the length of the cell cycle. Their results indicated that accumulation of BDD is possible in non-proliferative cells with a high transcription level [66, 67]. We know that NO/RNS concentrations, which stimulated cell proliferation, cannot damage DNA directly, but can block error-free HRR of DSB and lead to accumulation of mis-repaired DSB lesions. In our recent work, we demonstrated that NO•, generated in macrophages, initiated block of BRCA1 protein expression and subsequent inhibition of HRR in bystander cell lines (Fig. 2.6) [1]. Hence, NOS activation and overproduction of NO/RNS under chronic inflammation or tumor inflammatory microenvironment affect not only cells with activated NOS, but also bystander, not activated, cells. This effect can be determined as a "field effect" of genomic instability, maintained by the active inflammatory microenvironment. The similar "field effect" in cancer biology, related to the stromal production of reactive oxygen species (ROS) and NO/RNS, was recently proposed by another group of investigators [68]. They proposed that such "field effect" could be related not only to the immune cells, but also to the adjacent fibroblasts, as an additional source of ROS and NO/RNS. eNOS- expressing fibroblasts have the ability to downregulate Cav-1 (endogenous eNOS inhibitor) and induce mitochondrial dysfunction in adjacent fibroblasts that do not express eNOS. As such, the effects of stromal oxidative stress can be laterally propagated, amplified, and are effectively "contagious": spread from cell- to-cell like a virus, creating a ***"mutator field",*** promoting widespread genomic instability [68].

If we accept NO/RNS-depended mutagenesis as a completely stochastic process, we can postulate that the efficacy of mutagenesis (ME) and carcinogenesis in such "mutagenic filed" depends on the area of the field (FA), the strength of NO/RNS-maintained genomic instability (SGI), and the duration of this field maintenance (FD):

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