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Nitric oxide and interactions with reactive oxygen species in the development of melanoma, breast, and colon cancer: A redox signaling perspective

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ABSTRACT

Cancer development is closely related to chronic inflammation, which is associated with identifiable markers of tumor progression, such as uncontrolled cell proliferation, angiogenesis, genomic instability, chemotherapeutic resistance, and metastases. Redox processes mediated by reactive oxygen species (ROS) and nitric oxide (NO) within the inflammatory tumor microenvironment play an essential role in directly influencing intercellular and intracellular signaling. These reactive species originating in the cancer cell or its microenvironment, mediate the epithelial-mesenchymal transition (EMT) and the mesenchymal-epithelial transition (MET). However, intracellular interactions between NO and ROS must be controlled to prevent cell death. Melanoma, breast, and colon cancer cells have developed a mechanism to survive and adapt to oxidative and nitrosative stress. The mechanism involves a spatial-temporal fine adjustment of the intracellular concentrations of NO and ROS, thereby guaranteeing the successful development of cancer cells. Physiological concentrations of NO and supra physiological concentrations of ROS are prevalent in cancer cells at the primary site. The situation reverses in cancer cells undergoing the EMT prior to being released into the blood stream. Intracellular supra physiological concentrations of NO found in circulating cancer cells endow them with anoikis resistance. When the anoikis-resistant cancer cells arrive at a metastatic site they undergo the MET. Endogenous supra physiological concentrations of ROS and physiological NO concentrations are prevalent in these cells. Understanding tumor progression from the perspective of redox signaling permits the characterization of new markers and approaches to therapy. The synthesis and use of compounds with the capacity of modifying intracellular concentrations of NO and ROS may prove effective in disrupting a redox homeostasis operative in cancer cells.

1. Introduction

1.1. Epithelial-mesenchymal and mesenchymal-epithelial transitions and tumor progression

Cancer is the term used to describe a set of more than 200 diseases characterized as defects in tissue growth regulation resulting from mutations in genes that regulate cell proliferation and differentiation [1,2]. The World Health Organization estimates that approximately 27 million new cases of all cancers will be diagnosed by 2030, resulting in 17 million deaths and 75 million people affected by this disease, and that this increase will primarily affect developing countries.

A relatively high incidence of melanomas, breast and colon cancers have been determined in the Brazilian population. An increase in the

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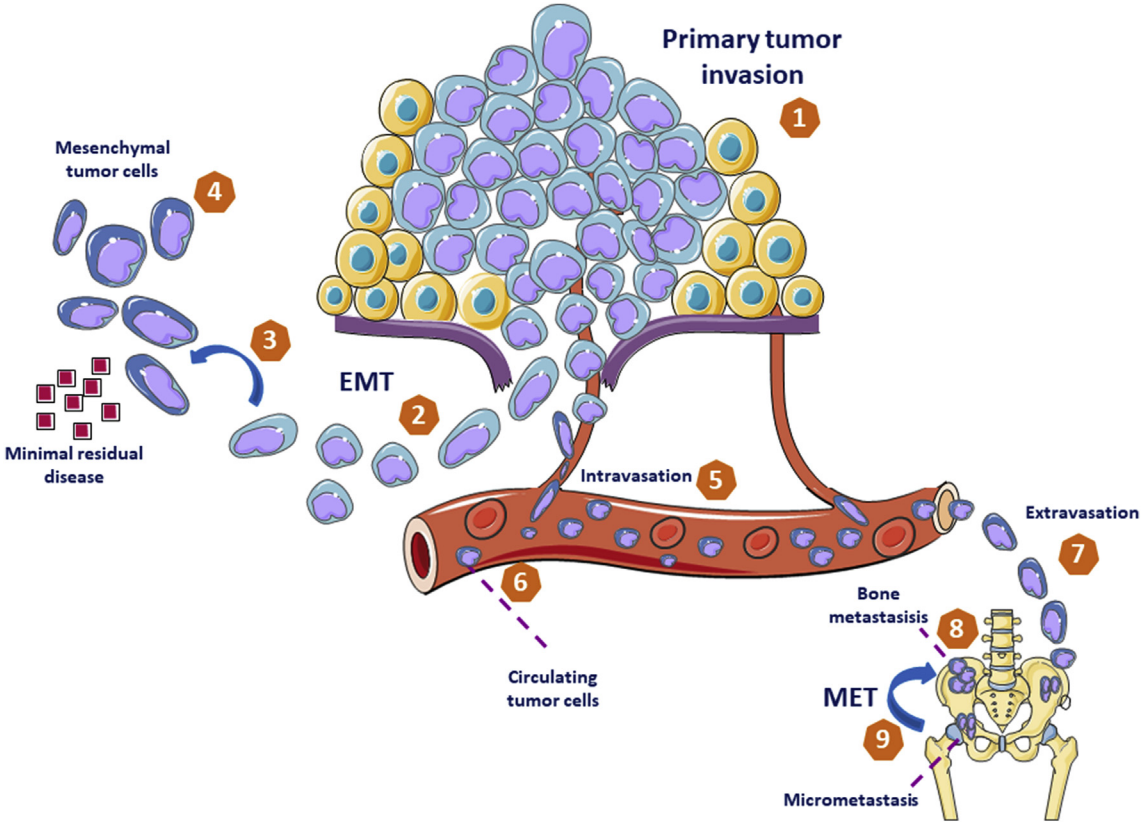

Fig. 1. Tumor progression and the key events in the epithelial-mesenchymal (EMT) and the mesenchymal-epithelial (MET) transitions. (1,2) Epithelial carcinomas undergo the EMT to escape the tumor mass, resulting in (3) minimal residual disease. (4) Re-epithelialization is necessary to develop a new malignant process. (5,6) Cells that undergo the EMT are de-differentiated and well adapted to perform intravasation and maintain circulation. (7) This fact assists in the extravasation process. (8) In this de-differentiated stage, cells can remain as micro-metastases in the bone marrow. (9) Reversal of the epithelial phenotype through the MET may occur, promoting the formation of macro-metastases and the death of osteoblasts. (Figure was elaborated using the source: Servier Medical Art. https://smart.servier.com).

number of cases of these three types of cancers has been estimated in Brazil, for the biennium 2018–2019, and, according to the numbers: a) 17,380 new cases in men and 18,980 new cases in women of colon cancer, with an estimated risk of 16.83 and 17.90 new cases per 100,000 men or women, respectively; b) 59,700 new cases of breast cancer, with an estimated risk of 56.33 new cases per 100,000 women; c) 2920 new cases in men and 3340 new cases in women of melanoma, with an estimated risk of 2.82 and 3.16 new cases per 100,000 men or women, respectively [3].

Melanoma, breast, and colon cancers share with the other cancers a common mechanism related to their initial transformation and progression towards metastasis. Malignant transformation may occur through the expression of new oncogenes, over expression and de-regulated activation of proto-oncogenes and down regulation or inactivation of tumor suppressor genes. Typically, cumulative changes in these genes are required for the transformation of a normal cell into a cancer cell [2,4]. Metastasis occurs when cancer cells simultaneously detach from the primary tumor by decreasing cell-cell interactions. This results in cellular migration and invasion into adjacent and distant organs [5].

The first transition is the epithelial-mesenchymal transition (EMT), in which a metastatic cell of epithelial origin expresses a set of genes typically expressed in connective tissue cells. The EMT is a de-differentiation process, where cancer cells of epithelial origin, become less differentiated and acquire a mesenchymal, fibroblast-like phenotype [2]. The EMT results from changes in protein expression patterns and transcriptional events in response to specific stimuli leading to important phenotypic changes [6]. It is an important step in tumor progression because it confers stem cell properties to the cancer cells and enables their invasion into the bloodstream. During the EMT, E-cadherin expression is transcriptionally repressed, and the expression levels of proteins associated with the mesenchymal phenotype, such as *N*-cadherin and vimentin, increase [7,8]. In addition to these proteins, a number of transcription factors, NFκB, SOX4, Snail, Slug, and Twist1, play an essential role in EMT activation [9,10].

Developmental signaling pathways stimulated by TGF-β, Notch, and Wnt have been associated with progression to the EMT. Progression to the EMT is also regulated by the EGFR, Src kinase, Ras/ERK1/2 MAPK, and the phosphatidylinositol-3 kinase (PI3K)/Akt canonical signaling pathways, whose constitutive activation, as occurs in cancer cells, up regulates the transcription factors associated with the EMT [8,11].

The occurrence of the EMT transition is an important step in tumor progression that precedes the spread of cancer cells into the bloodstream. Most of these mesenchymal de-differentiated cells are eliminated by the shear forces exerted on them by the blood flow and the actions of the host's immune system. Only 0.01% of the total cancer cells that have undergone the EMT and are dissociated from the primary tumor mass, survive the migration through the bloodstream and reach the remote site of metastasis [5]. Once arriving at metastatic sites, mesenchymal cancer cells revert to the epithelial phenotype to complete colonization and initiate metastasis.

EMT-promoting factors, particularly TGF-β and Twist1, induce a reversible EMT to form epithelial metastases [12,13]. The expression of the low molecular weight glycoprotein podoplanin (PDPN) [14], is associated with the down regulation of E-cadherin, an important marker of the EMT [15,16]. PDPN binds to the membrane-associated protein CLEC-2 on platelets in the bloodstream, thereby inducing platelet aggregation and facilitating tumor embolization. PDPN-mediated tumor cell-induced platelet aggregation promotes the extravasation step of metastasis [17]. TGF-β, which is released during platelet

aggregation induced by PDPN in bladder squamous cell carcinoma cells, may promote the formation of epithelial metastases through reversion of the EMT [12,18].

The mesenchymal-epithelial transition (MET), the reverse process of the EMT, is important for cancer cell metastasis dynamics [6,19]. Increasing E-cadherin expression is an important marker of the reversion of the EMT, and is indicative of the occurrence of the MET. MET markers have been detected in human synovial sarcomas, and in human ovarian carcinoma [20,21]. Because the MET is a re-differentiation process, it plays an essential role in the processes of recognition, colonization and adaptation of the metastatic cell to the microenvironment of the local/regional or distant metastatic site.

A schematic view of the general development of cancer cells beginning at the site of origin, passing through the EMT and the MET to become an established metastasis, is shown in Fig. 1.

1.2. Tumor microenvironment factors involved in tumor development

Cancer is frequently described as “a wound that does not heal”, suggesting that cancer promotes a chronic inflammatory condition that does not resolve. This condition is associated with the main characteristics of tumor progression, such as disordered proliferation, angiogenesis, genomic instability, chemo-resistance, and metastasis [22]. A highly inflammatory environment is produced not only by cancer cells, which secrete pro inflammatory cytokines, but also by other components of the tumor microenvironment. These components include immune system-infiltrating cells with an immunosuppressive profile, vascular endothelial cells responsible for tumor angiogenesis, and components of the extracellular matrix capable of generating and storing pro-inflammatory products. Cancer-associated fibroblasts have been included in this list as they are subverted in this environment and produce conditions conducive to tumor progression [23].

Reactive oxygen species (ROS) are mediators and modulators of various cellular signaling processes that govern the development of tumors [24,25]. In the last twenty years, the importance of nitric oxide (NO), another important oxidant generated chronically at inflammatory sites has been increasingly recognized. The chronic generation of NO and ROS plays an essential role in maintaining oxidative/nitrosative stress in tumors [26–28].

There is a growing body of evidence showing the participation of NO synthases, in particular the inducible isoform, and the NADPH oxidase isoforms 1–5, as sources of NO and ROS, respectively, in cancer development [28,29]. Both reactive species may act on the EMT and the MET, and are produced by cancer cells and by other components of the tumor microenvironment [30,31] (Fig. 2).

The impact of NO on tumor progression is best understood by knowing the dual role played by NO as a function of its concentration. Physiological and intermediate concentrations of NO are pro-carcinogenic, whereas supra physiological concentrations are anti-carcinogenic [32].

Another issue to be addressed is the interaction between NO and ROS and its significance in tumor biology. A concept of a reactive species interactome involves the interaction between NO and ROS, among others. Such interactions are potentially determining factors regarding cellular homeostasis [33].

Understanding in depth the role played by NO and its interaction with ROS in the progression of melanoma, breast, and colon cancer may provide important information on how to design new therapeutic strategies based on the redox state of the cancer cell. A discussion addressing the role of NO in cancer therapeutics is also provided.

2. Nitric oxide synthases and their relevance in the development of melanoma, breast, and colon cancer

NO is a gaseous free radical with signaling properties and relative stability in biological systems. Stability is derived from reactions of dimerization or from rapid reactions with oxidizing and/or reducing compounds. NO reacts with organic compounds containing nucleophilic groups such as alcohols (ROH), thiols (RSH) and amines (RR'NH) to generate RO-NO, RS-NO, and RR'N-NO, respectively [34]. Being a free radical, NO strongly reacts with paramagnetic substances, such as iron, O_2, and O_2^-. These reactions characterize the direct effects of NO, while some of its products, such as nitrogen oxides, are responsible for the indirect effects of NO [35].

Almost all normal and tumor cells produce NO using heme-enzymes from the family of NADPH cytochrome P450 reductases, known as NO synthases (NOS) [36]. In mammals, three isoforms have been characterized: NOS1 (neuronal) and NOS3 (endothelial), which are constitutively expressed, and NOS2, which is the inducible isoform and was initially characterized in macrophages. All isoforms require L-arginine as substrate for the synthesis of NO. Although they present common functions, NOS isoforms are codified by different genes and have different characteristics [37] (Fig. 3).

Melanoma, breast and colon cancer cells may express more than one NOS isoform. NOS1 expression is further induced by de novo expression in melanomas at their earliest stages. An additional increase in NOS1 expression has been detected as melanomas continue to develop. Being highly recurrent in biopsies of primary malignant melanoma, 83% of the cases have shown NOS1 in the epidermal compartment [38]. Epidemiological studies have provided evidence of the relevance of NOS1 polymorphisms in melanoma and colorectal cancer risk [39,40]. Expression of NOS1 in melanomas is associated with the constitutive overproduction of NO. This promotes melanoma cell invasion and proliferation, thus allowing for the next stage in the development of metastases [41].

In biopsies of malignant melanomas, NOS3 expression is correlated with the expression of VEGF; it is likely that NOS3 plays a role as mediator of VEGF induced angiogenesis [42]. NOS3 expression is also directly correlated with the expression of estrogen receptors (ERs) in breast carcinoma cells [43–46], suggesting an important role for NOS and NO in the etiology of ER positive breast cancer.

Epidemiological studies have shown the association between polymorphisms of NOS3 and cancer risk. Different NOS3 polymorphisms described in breast and colorectal tissue are associated with the susceptibility to develop breast and colon cancer [45–48].

Among the three isoforms, NOS2 expression has been the most consistently associated with the development of melanoma, breast, and colon cancers [49–51]. Cytokines that are secreted into the tumor microenvironment and stimulate NO production by NOS2 are tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β) and interferon γ (INF-γ). Other stimuli include hypoxia, phorbol esters, lipoarabinomannan and lipopolysaccharide from Gram-negative bacteria [52].

In addition to the production of NO resulting from activation of the various components of the tumor microenvironment, the importance of endogenous NO production by melanoma, breast, and colon cancer cells has been demonstrated [53–58].

NOS2 up regulation occurs in conditions of fetal bovine serum removal from the cell culture medium. This condition stimulates NO production in MDA-MB-231 triple-negative breast cancer (TNBC) cells which are negative for the expression of estrogen, progesterone and Her 2/neu receptors [55].

NOS2 and NOS3 promote the migration and invasive capacity of breast and colon cancers by activating soluble guanylate cyclase and the ERK1/2/MAPK pathway [59]. NOS2 is expressed in approximately 70% of all types of breast cancer [60]. In TNBC patients, NOS2 is associated with a negative prognosis and the NOS2/NO pair is an important determinant of decreased survival [61].

Normal and cancerous breast tissue express differential levels of the NOS2 and NOS3 isoforms with increasing levels of both isoforms in cancerous breast tissues [48,62]. On the other hand, NOS1 was equally expressed in normal and cancerous breast tissue [48]. A positive

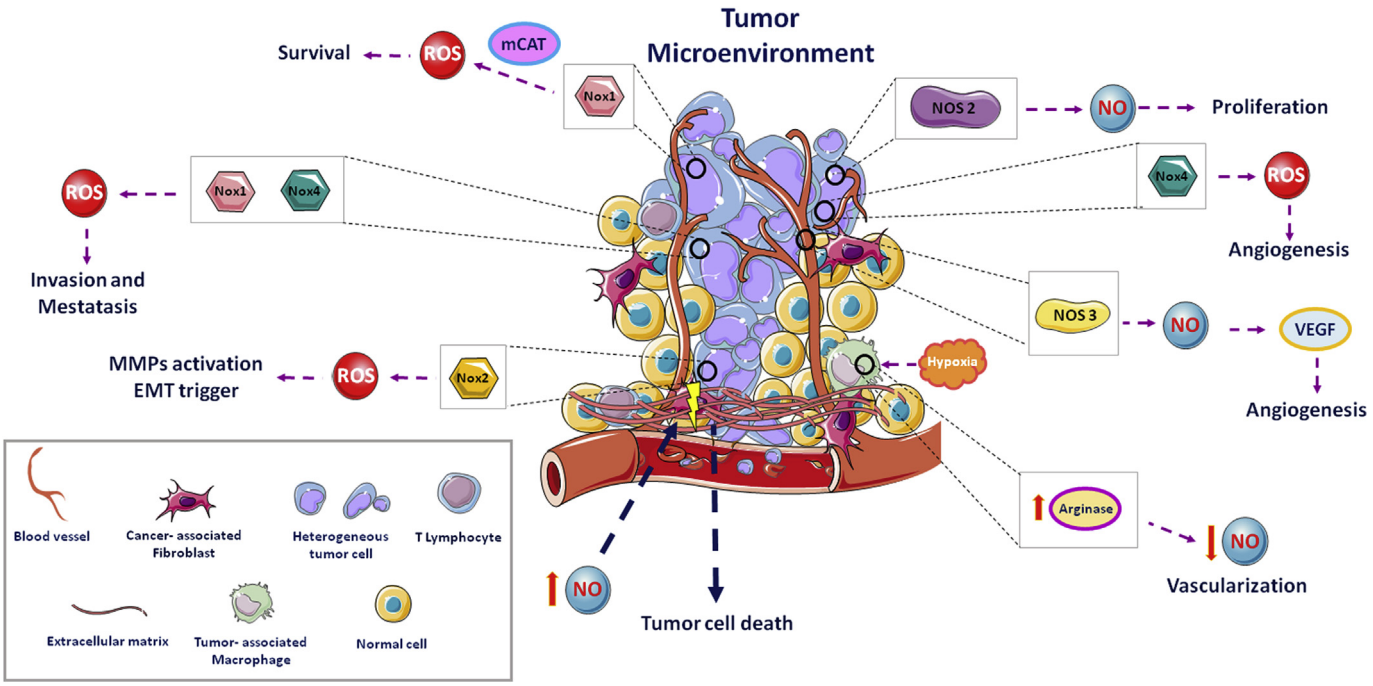

Fig. 2. Nitric oxide, reactive oxygen species, and the tumor microenvironment. Tumor microenvironment elements communicate with each other and use NO and ROS to stimulate signaling pathways that will promote more aggressive tumor phenotypes. The NOS2 and NOS3 isoforms participate in angiogenesis with the mediation of VEGF. Angiogenesis can be stimulated by ROS derived from NOX4 activity. Tumor-associated macrophages contribute to tumor development by over-expressing Arginase and producing physiological levels of NO. Cytotoxic macrophages promote cancer cell death by producing supra physiological concentrations of NO and ROS. ROS produced by NOX1, 2, and 4 promote survival, metalloproteinase (MMP) activation, trigger the EMT, and metastasis. (Figure was elaborated using the source: Servier Medical Art. https://smart.servier.com).

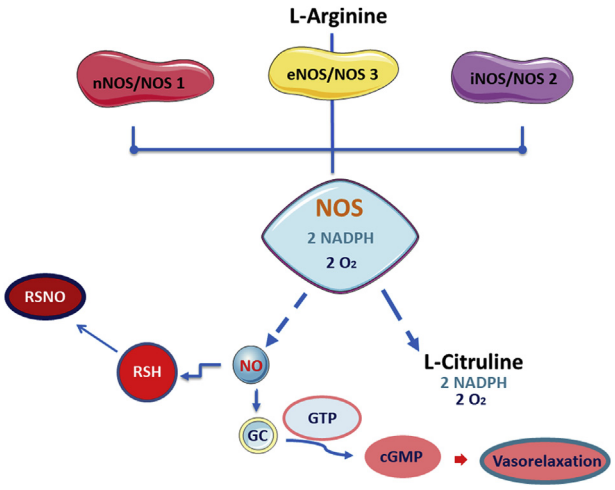

Fig. 3. NOS catalyzed biosynthesis of NO and its mechanism of action. NO is produced by three isoforms of NOS, NOS1, NOS2 and NOS3 that catalyze the oxidation of L-arginine to L-citrulline. NO activates the soluble guanylate cyclase enzyme with the production of the second messenger cGMP that initiates cellular signaling. NO also modifies proteins through s-nitrosylation, (reaction between NO and the thiol group of a specific cysteine residue of intracellular proteins), triggering cellular signaling. (Figure was elaborated using the source: Servier Medical Art. https://smart.servier.com).

correlation exists between NOS2 expression, histological grade, lymph node status, and the clinical stage of breast cancer biopsies [48,62].

Analogous to the situation with MDA-MB-231 TNBC cells, removal of fetal bovine serum from cultures of SW480 and SW620 human colon cancer cell lines elevate the expression levels of NOS2 with increasing endogenous production of NO [57]. Among human cancers, colon cancer shows the best correlation with the presence of chronic inflammation. NO produced by NOS2 at inflammatory sites stimulates the progression of colon cancer [63]. NOS2 is expressed in 50–60% of colon cancer patients and those with high NOS2 expression have a worse prognosis than those with low NOS2 expression [63,64]. NOS2 expression is absent in colon epithelial sections of healthy individuals, is low-to-intermediate in patients with chronic colitis and is high in patients with colon cancer [65]. Low NOS2 expression is found in the initial stages of colon cancer [53]. These observations suggest a direct relationship between NOS2 expression and the progression of colon cancer. However, the role of NO and NOS2 during other stages of the development of colon cancer remains unknown.

NOS2 expression is higher in melanoma cells than normal melanocytes and its expression increases with increased risk of metastasis [66,67]. The NO endogenously produced by some types of human melanomas determines their survival. Increased NOS2 expression and the presence of nitro-tyrosine in cancer cells of patients with melanoma are related to the low survival rate of these patients, suggesting that NO plays an important role in resistance to therapy in melanoma [68]. An increase in intracellular NO levels in cisplatin-resistant cells is correlated with the inactivation of the enzymes prolyl-hydroxylase-2 and caspase-3, resulting in cell resistance to chemotherapeutic agents [69]. In B16F10 murine melanoma cells, blockade of β3-adrenergic receptors inhibits the proliferation and activation of apoptotic pathways by the sequential inhibition of NOS2 expression and a subsequent decrease in NO production. When β3-adrenergic receptors are activated, the opposite effect is observed, which is attributed to an increase in NOS2 expression followed by an increase in NO. Although the constitutive isoforms, namely NOS1 and NOS3, also participate in this process, NOS2 activity is predominant [70].

Elevated expression and increased activity of the NOS2 isoform is a determining factor for the poor prognosis in patients with TNBC, colon cancer, and melanoma [71,72].

3. *S*-nitrosylation and pro-oncogenic signaling mediated by nitric oxide

The dual role played by NO in signaling in normal and cancer cells is related to its local concentration, to the time of exposure, to the cell type, and to its interaction with other reactive species [32,73,74]. Intracellular NO in the pM to nM concentration range are produced by NOS1 and NOS3 in neuronal and endothelial cells and are associated with synaptic connections and vascular cell relaxation/proliferation, respectively [75–77]. In this review, concentrations of NO within the range of pM to low nM are defined as physiological concentrations, while higher NO concentrations, ranging from high nM to μM, produced by NOS2 in cancer cells and in macrophages, are defined as intermediate to supra physiological concentrations. This concentration range of NO has been associated with pro carcinogenic and tumoricidal activities [78,79].

Concentrations of NO ranging from pM to low nM generate signaling through nitrosylation of the regulatory ferrous heme of the soluble isoform of the enzyme guanylyl cyclase. This action results in the production of the intracellular second messenger, cGMP [80]. The occurrence of cGMP-independent NO signaling is associated with NO concentrations ranging from low nM to μM [28]. Within this range of concentrations, the modification of cysteine residues in proteins, referred to protein *S*-nitrosation is the best characterized. Although the term that defines the chemical reaction between NO and thiols is *S*-nitrosation, *S*-nitrosylation is the term that describes the biological function of this modification operating in proteins.

S-nitrosylation is defined as the addition or a transfer of a nitroso group moiety to a thiol group of a reduced cysteine forming an s-nitrosothiol (SNO) in proteins and/or peptides such as GSH. The formation of SNO in biological systems may occur through four major pathways: (a) The transition metal catalyzed direct addition of NO to a thiol; (b) The reaction with O_2 to form two strong nitrosylating species, peroxynitrite radical ($^{\cdot}ONOO$) and N_2O_3; (c) The recombination of NO and thiyl radicals; (d) The transfer of SNO groups to free thiols referred to *trans*-nitrosylation [37].

S-nitrosylation of specific cysteine residues has been described in more than 1000 proteins in different biological settings [81–84]. Protein *S*-nitrosylation is an evolutionary conserved posttranslational modification [85,86]. It regulates protein activity, stability, localization, and protein-protein interactions. In normal and cancer cells, protein *S*-nitrosylation can be derived either from NOS activities or from the exposure of the cells to the low molecular weight *S*-nitrosothiol (SNO) *S*-nitrosoglutathione (GSNO) [37,81].

By analogy to protein kinases, *S*-nitrosylated proteins can *trans*-nitrosylate other proteins and the newly formed nitrosylases can propagate SNO-based signals. Like other posttranslational modifications, *S*-nitrosylation is reversible, with protein nitrosylases and denitrosylases controlling the intracellular steady state levels of SNO [86]. Two denitrosylases of major importance in signaling have been characterized, GSNO reductase and Thioredoxin-1 (Trx-1). GSNO reductase regulates GSNO levels and indirectly regulates protein *S*-nitrosylation [87]. Trx-1 functions as a denitrosylase by removing NO from s-nitrosylated cysteine residues in both the GSNO peptide and in signaling or structural proteins [88–91]. Tightly controlled levels of *S*-nitrosylated oncogenic proteins are essential in regulating the pro- and anti-oncogenic activities of these proteins [92]. Elevated Trx-1 expression in cancer cells maintains the level of SNO that up regulates signaling pathways associated with survival, an important feature of disordered growth in cancer cells [93].

Although the role played by *S*-nitrosylation in signaling has been described, the other reactions of NO that compete with *S*-nitrosylation must be considered. These reactions include the binding to Fe^{2+} heme groups in heme proteins such as soluble guanylyl cyclase with a rate constant value higher than $10^8\,M^{-1}\,s^{-1}$ [94], and the recombination with O_2^- to form peroxynitrite anion ($ONOO^-$) with a rate constant of $4.3 \times 10^9\,M^{-1}\,s^{-1}$ [95]. Peroxynitrite anions can modify proteins through nitration of tyrosine residues and tyrosine nitration can compete with *S*-nitrosylation for intracellular NO-mediated signaling [74]. In human colon cancer tissue, NOS2 and nitrotyrosine expression are elevated when compared to that in normal colon tissue. These findings are associated with tumor-related immunosuppression [96].

The experimental evidence on the role of tyrosine nitration in signaling is not as abundant as that for *S*-nitrosylation. However, protein tyrosine nitration may target different signaling proteins and thereby promote cancer development [74,96].

3.1. *S*-nitrosylation and protein phosphorylation in oncogenic signaling pathways

Many of the genes commonly mutated in cancer encode components or targets of the Src kinase, EGFR, PI3K/Akt and Ras/ERK1/2 MAPK pathways [11]. A cross talk between protein *S*-nitrosylation and protein phosphorylation is associated with the NO-dependent activation of signaling proteins involved in the Ras/ERK1/2 MAPK, PI3K/Akt, Src kinase and EGFR oncogenic signaling pathways [60,71,77,81,97–100].

S-nitrosylation of Cys118 in Ras and Cys498 in Src kinase mediated by physiological NO concentrations promotes the activity of these signaling proteins, stimulating Ras GDP-GTP exchange and tyrosine phosphorylation of Src kinase [99–101]. NO derived from the NO donors, Sodium nitroprusside (SNP) and *S*-Nitroso-*N*-acetylpenicillamine (SNAP), stimulate tyrosine phosphorylation of proteins of the signaling pathway initiated by EGFR activation [102]. These proteins are the protein tyrosine kinases, focal adhesion kinase (FAK), and Src kinase, and the Ser/Thr protein kinases, ERK1/2 MAPK [103].

Ras is an important initiator of oncogenic signaling. *S*-nitrosylation of the cysteine residue, Cys118 (part of a conserved NKCD amino acid sequence close to the guanine nucleotide-binding domain), activates Ras when cells are exposed to different NO donors [104]. Ras *S*-nitrosylation in endothelial cells is the first step of a signaling cascade consisting of the Raf-1/MEK/ERK1/2 MAPK protein kinases [97]. Ras *S*-nitrosylation and ERK1/2 MAPK activation, by the *S*-nitrosothiol, SNAP, are the initial steps of a sequence of signaling events (phosphorylation of transcription factors and induction of cyclin expression), culminating in cell cycle progression [98]. Ras activation by *S*-nitrosylation recruits Rac1 and PI3K, thereby stimulating cell migration [105]. GSNO induces Ras activation in HeLa cervical cancer cells by *S*-nitrosylation in a compartmentalized process with an initial stage of the process occurring in the plasma membrane and a second stage occurring in the Golgi complex [106].

Src kinase activity is up regulated by *S*-nitrosylation [99,100]. Src interacts with the signaling proteins of the focal adhesion complex, FAK and p130Cas, in mouse embryonic fibroblasts exposed to SNAP at concentrations that release physiological levels of NO. These interactions are accompanied by Src kinase *S*-nitrosylation and stimulation of the migratory capacity of these fibroblasts, which is associated with the expression of another element of the focal adhesion complex, protein tyrosine phosphatase α (PTPα) [99]. *S*-nitrosylation and Src kinase activation is observed in MCF-7 breast cancer cells, characterized by the absence of PTPα expression, when these cells are incubated with NO donors or stimulated with estrogen [100,107]. This is corroborated by the finding that the NO donor, diethylene-triamine–(DETA)-NONOate, induces EGFR and Src kinase *S*-nitrosylation and tyrosine phosphorylation in human TNBC cells [60,108].

3.2. Nitric oxide mediated-signaling and the epithelial-mesenchymal transition (EMT)

NO-mediated activation of the oncogenic signaling pathways discussed in the previous section strongly suggests that a connection exists between this activation and the induction of the EMT. However, direct evidence of this connection still is a matter of debate, since the effects of

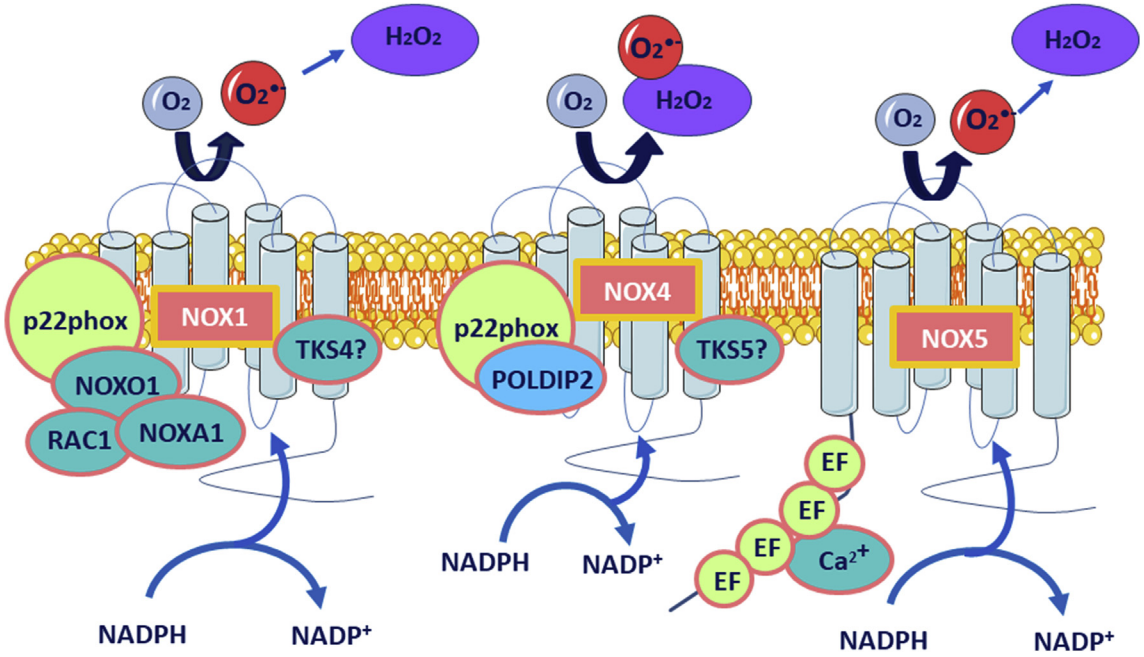

Fig. 4. Structures and molecular organization of NOX1, 4, and 5. These isoforms are predominantly expressed in cancer cells. (Figure was elaborated using the source: Servier Medical Art. https://smart.servier.com).

physiological and supra physiological concentrations of NO may promote and inhibit the EMT, respectively [109–112].

Continuous exposure of certain cancer cell types to growth factors like EGF, turn on the EGFR, Src kinase, Ras/ERK1/2 MAPK, and PI3K/Akt pathways driving the EMT in certain contexts, generally under conditions in which these pathways are hyper-activated together with other pathways implicated in the EMT [11,113]. EMT induction involves the acquisition of invasive and migratory phenotypes and both phenotypes are also induced by physiological concentrations of NO [32].

High expression of NOS2 is associated with a poor clinical outcome in patients with TNBC [61,109]. After targeting NOS2 using a specific inhibitor (1400 W) or two general NOS inhibitors (L-NMMA and L-NAME), the rate of cancer cell proliferation is decreased and the EMT transcription factors, Snail, Slug, Twist-1, and Zeb-1 are inhibited [109]. TNBC cells and colon cancer cells exposed to hydrodynamic shear stress conditions that mimic the forces exerted on circulating cancer cells undergo an EMT-like transition that is dependent on ROS and NO production [110].

In A431 and A459 epithelial cancer cell lines from skin and lung origins respectively, the concerted action of EGF, inflammatory prostaglandin E-2, and NOS2 promote changes in cell phenotype as demonstrated by the acquisition of stem-cell features and activation of the EMT [114]. Repeated sub-optimal treatment with the photosensitizer pheophorbide α in prostate cancer cells PC3, promotes the EMT through generation of physiological concentrations of NO and the activation of the PI3K/Akt signaling axis [115].

Supra physiological levels of NO generated by millimolar concentrations of NO donors target the EMT associated transcription factors NFkB, Snail, and Y1Y1. NO-mediated down regulation of these transcription factors results in inhibition of the EMT [reviewed in 111].

Enhanced survival of circulating cancer cells that undergo the EMT, involves the acquisition of resistance to anoikis, a form of cell death that occurs when cells lose contact with the extracellular matrix and with neighboring cells [116]. Long-term (six days) exposure of the non-small-cell lung cancer cell line H23 to physiological concentrations of NO-derived from DETA-NONOate maintains cell viability. These cells are resistant to anoikis and have increased levels of the EMT markers vimentin and Snail [117].

HeLa cells and B16F10/Nex8H metastatic murine melanoma cells maintained in suspension to simulate anoikis conditions have been exposed to supra physiological concentrations of NO derived from the NO donors SNP and DETA-NONOate. A period of exposure of 24 h to both NO donors results in enhanced survival of both cell lines, which is associated with the up regulation of caveolin-1 expression, s-nitrosylation and tyrosine phosphorylation of Tyr416 on Src kinase, and down regulation of the pro-apoptotic proteins, Bim and cleaved caspase-3 [118].

Supra physiological NO levels apparently are necessary for maintaining cancer cell viability while these cells are detached from the ECM and from neighboring cells [118]. NO levels must decrease to permit the mediation of ROS [119] in establishing the necessary conditions of cells to reattach to a permissive surface, spread, and proliferate [118].

In cancer cells, an intracellular imbalance between NO and ROS must be achieved to maintain a redox based homeostasis and permit their development. The evidence gathered to support this assumption is presented and discussed in the following sections.

4. Interactions between nitric oxide and reactive oxygen species in pro-oncogenic signaling

4.1. Brief overview on reactive oxygen species signaling in cancer

The “theory of the participation of free radicals in cancer” states that oxidative stress is associated with cell transformation [120]. This theory originated from an evaluation of evidence that high H_2O_2 and O_2^- production in cancer cells stimulates disordered proliferation and immortalizes these cells. It was suggested that the NADPH oxidase (NOX) enzyme/enzymatic complex is not exclusively expressed in neutrophils and may account for elevated ROS production in cancer cells [121].

Subsequent discoveries have corroborated this original hypothesis and other studies have demonstrated that high ROS levels are also produced by exacerbated activity of the mitochondrial respiratory chain in cancer cells [122]. The existence of a crosstalk between NOX and the mitochondrial respiratory chain was found in breast and

ovarian tumors [123].

Seven NOX isoforms have been isolated and characterized. NOX 1–5, which primarily generate O_2^- (although NOX4 can produce O_2^- and H_2O_2 with the same efficacy) are associated with the plasma membrane and with the endomembrane system; and two enzymes, DUOX 1 and 2, which primarily produce H_2O_2 [74]. Mechanistic and clinical studies highlight the key role of NOX isoforms 1, 2, 4 and 5 in several types of tumors [30]. Fig. 4 shows a schematic representation of the structures and molecular organization of NOX 1, 4 and 5.

Of particular interest for the ROS-mediated signaling events associated with oncogenesis are the NOX1 and NOX4 isoforms [30].

NOX1 activity **is** associated with a sustained extracellular production of O_2^- and H_2O_2 which is controlled by activated oncogenes. This is a characteristic feature of cancer cells and represents a very effective mechanism of autocrine stimulation of tumor cells [124–126]. A membrane-associated Catalase (mCAT) maintains the viability of cancer cells expressing NOX1, in their early stages of development [127].

NOX4 has been identified in the endoplasmic reticulum and in mitochondrial and nuclear membranes. The location of NOX4 in the cell nucleus raises the possibility of direct DNA and nuclear protein alterations through *in situ* ROS generation [128,129].

Signaling mediated by NOX/ROS, specifically by H_2O_2, is indirect and translates into the transient oxidation of cysteine residues at the active site of phosphatases. Transient phosphatase inactivation results in the hyper-phosphorylation and activation of protein kinases regulated by phosphatases and in the activation of other signaling proteins located downstream in the signaling cascade [130]. Phosphatase inactivation by ROS produced by NOX is associated with highly efficient survival pathways in several types of cancer cells [30].

4.2. Pro-oncogenic signaling associated with the interaction between nitric oxide and reactive oxygen species

Evidence of the occurrence of NO- and ROS-mediated signaling processes in cancer cells is mounting [70,131,132]. However, the high reactivity between NO and O_2^- which results in the formation of ONOO- a reactive and highly cytotoxic species [133], would prevent the occurrence of signaling by NO and ROS in the same cell compartment and at the same cancer cell stage.

Production of NO and ROS in appropriate concentrations by stromal cells in the tumor microenvironment is an important feature associated with tumor development. Macrophages are important components of the tumor microenvironment. Tumor-associated macrophages (TAM) participate in the inflammatory response generated from the interaction between cancer cells and stromal cells. The action of TAMs may either suppress or stimulate tumor development. Macrophage cytotoxic activities associated with tumor suppression are related to the production of supra physiological concentrations of NO and ROS [134].

To stimulate tumor development, TAMs are reprogrammed to generate physiological concentrations of NO through the over-expression of the enzyme Arginase-1 (Arg-1) and the down regulation of NOS2 expression [135].

Over-expression of Arg-1 in TAMs is related to the effects of lactic acid produced by aerobic and anaerobic glycolysis and released by cancer cells in the tumor microenvironment. Lactic acid stimulates the expression of VEGF by TAMS through the induction of HIF-1α [136]. The up regulation of VEGF and Arg-1 in TAMs might support tumor growth by production of physiological concentrations of NO that could induce angiogenesis and cancer cell proliferation [136].

ROS positively regulate the angiogenic switch in cancer cells by enabling increased VEGF production and the up regulation of HIF-1α expression [30,137]. NOX2-derived ROS can promote the recruitment of cancer-associated fibroblasts, the activation of matrix metalloproteinases, and the initiation of the EMT [138]. Although ROS are inducers of the EMT, they are not capable of sustaining the EMT [139,140]. It is likely that the EMT depends on NO and ROS interactions for stable induction (**Fig. 2**). The interactions between NO and ROS produced by the cancer cell are very important in determining a condition of homeostasis that will define which reactive species plays a pro-oncogenic role during tumor progression.

The high NOX 1–5 expression levels detected in cancer cell lines at early and late stages (primary tumor and consolidated metastases, respectively) suggest that ROS may play a key role in both stages [30].

An important aspect of the pro-oncogenic role of ROS is the high mutagenicity of these species. The oxidation of DNA bases by ROS results in the formation of 8-hydroxy-deoxyguanosine adducts, which may lead to mutations and consequent cell transformation [141].

This is corroborated by early studies reviewed by Deichman [142]. In these studies transformation of normal cells *in vitro or in vivo*, spontaneously or induced by various oncogenes, with subsequent natural selection *in vivo* of their descendants, results in cancer cells expressing high levels of the antioxidant enzyme membrane-associated Catalase (mCAT). mCAT is highly expressed in more than 70 human cancer cell lines and the enzyme protects cancer cells against ROS-mediated apoptosis. Inhibition of mCAT in these cancer cell lines induces ROS-mediated apoptosis [143].

Reversible inhibition of mCAT by NO is achieved upon exposure of the enzyme to increasing concentrations of the radical [144]. Nitric oxide at low concentrations is oxidized to nitrite in a two-step reaction by Compound I of mCAT, which is formed through the reaction between cancer cell–derived H_2O_2 and mCAT [145]. When the production of cancer cell-derived NO is enhanced, mCAT is reversibly inhibited through formation of an mCAT-NO adduct [146]. At the initial stage of tumor development, control of the endogenous NO level is of major importance in maintaining cancer cell homeostasis, leading to prevention of ROS/NO-mediated apoptosis. Low concentrations of NO produced by constitutively expressed NOS2 in melanoma, breast, and colon cancer cells may represent an important factor for their survival at their initial stage of development [51,56,61].

The higher efficiency of NOS2 in generating NO as compared to the other isoforms and its role as the main source of NO as a pro-oncogenic signaling agent [50,71,72] appears inconsistent with tumor growth. Since supra physiological concentrations of NO may promote cell death, the high efficiency of NOS2 in generating NO must be counteracted to create a pro-oncogenic environment.

Alternative splicing of NOS2 mRNA has been proposed as a participant in the regulation of NOS2 expression and/or activity [147]. Cloning and characterization of alternative splicing variant S2 of NOS2 reveals that deletions of exons 8 and 9 prevent the formation of homodimers and the production of NO by this isoform [147]. A S3 variant characterized by the deletions of exons 9, 10, and 11 is associated with down regulation of NO production [148]. Modulation of NO synthesis by alternatively spliced variants could be achieved through the formation of heterodimers.

NOS2 alternative splicing expression levels are important in maintaining pro-oncogenic NO levels in human colon cancer cell lines. Using isogenic human colon cancer cell lines derived from either the primary tumor (SW480) or from a lymph node metastasis (SW620), it was shown that SW480 cells express significantly higher levels of the splicing variant S3 than SW620 cells. The expression of the NOS2 variant S3 in SW480 cells down regulates intracellular NO production [149].

The accelerated growth of the tumor mass at the primary site generates a heterogeneous population of cancer cells. Within this population a small group begins to undergo the EMT transition, which gives these cells greater capacity of migration and invasion [150].

The detection of high NOS2 expression and the association of the resulting increased NO concentration with enhanced cancer cell migration [28,71] and protection against cancer cell death by anoikis with supra physiological NO concentrations [118] suggests that NO is important in cell detachment from the primary tumor periphery, the invasion of lymph nodes, cancer cell embolisms and the entrance of

cancer cells into the blood stream. In this stage of tumor development, elevated intracellular NO levels might be accompanied by low intracellular levels of ROS. Corroborating our hypothesis, lower levels of ROS have been detected in the colon cancer cell line SW620 when compared to the levels found in the colon cancer cell lines, SW480 and HCT116, obtained from established liver metastasis [151].

As discussed in previous sections, NOS2/NO-mediated redox signaling in cancer cells results in direct NO activity mostly through *S*-nitrosylation of signaling proteins. Furthermore, the interaction between ROS and NO can be a determining factor for the occurrence of *S*-nitrosylation in cancer cells. In favor of this are the findings that an increase in intracellular ROS up regulates NOS expression in endothelial cells [152] and that ROS stimulate protein *S*-nitrosylation in serum-starved mouse embryonic fibroblasts exposed to H_2O_2 [153].

S-nitrosylation inactivates NOX4 in endothelial cells [154]; however nothing is known about a possible NO-dependent inactivation of this enzyme in cancer cells. Inactivation of NOX4 by *S*-nitrosylation in cancer cells undergoing the EMT could be of major importance in maintaining tumor redox homeostasis in this stage of tumor progression.

Another important aspect of the interaction between ROS and NO in tumor progression occurs during the switch from the EMT to the MET. A successful MET is associated with platelet aggregation induced by cancer cells which is mediated by the glycoprotein PDPN [17]. No direct effect of NO on PDPN expression or activity has been demonstrated [155]. However, NO plays a significant anti-aggregating role at supra physiological concentrations [156], suggesting that NO levels must decrease in cancer cells promoting aggregation of platelets. Conversely, ROS plays a pro-aggregating role [157,158], suggesting that ROS levels are elevated in cancer cells to promote platelet aggregation. Therefore, high ROS levels and reduced NO levels most likely reflect the redox state in metastasis formation at distant sites from the primary tumor.

Indirect evidence of this situation is apparent as observed in an HCT116 colon cancer cell line that has undergone the MET (Duke's D stage), as compared to HT29 cells, a colon cancer cell line at an earlier stage of development (Duke's C stage). Higher expression of the peroxiredoxin-1 antioxidant enzyme has been found in HCT116 cells as compared to HT29 cells [159].

5. Anti-oncogenic signaling mediated by nitric oxide

In the preceding section various arguments were presented in favor of a fine balance between the NO and ROS levels during the different stages of melanoma, breast, and colon cancers. A shift from the optimal situation derived from redox equilibrium established at different stages of development can be achieved by increasing the intracellular NO concentration in cancer cells. An NO donor-based cancer therapy can be designed by increasing the intracellular NO concentration using a number of strategies [160].

Three different strategies for increasing the NO concentration in melanoma, breast, colon, and other tumor cells are presented: (1) Endogenous NO production induced by adequate NOS2 stimuli in cancer cells; (2) Exogenous NO provision, resulting from the exposure of cancer cells to high concentrations of NO donors; (3) NO produced by stimulation of other cells in the tumor microenvironment and induction of immunogenic cell death.

5.1. Endogenous NO production by cancer cells: regulation of NOS2 expression and activity

NOS2 overexpression in colon cancer cells expressing wild-type p53 causes a decrease in tumor growth and an overexpression of vascular endothelial growth factor (VEGF) [53] with an increased intracellular concentration of NO [81]. High concentrations of NO produced by NOS2 in colon cancer cells induce apoptosis and inhibit tumor growth and metastasis in different animal models [53]. NO may inhibit metastases by inducing DNA damage, mutations and apoptosis in cancer cells. Over-expression of NOS2 in murine melanoma cells K-1735 is associated with apoptosis, suppression of tumorigenicity, and inhibition of metastases [161].

MDA-MB-231 TNBC cells implanted in immunosuppressed mice become sensitized to the chemotherapeutic agent docetaxel after treatment with a nanoparticle formulation for the delivery of NOS2 gene therapy [162].

5.2. Exogenous NO provision: nitric oxide donors as antitumor agents

Tumor progression inhibition resulting from increased intra-tumoral NO concentrations suggests that NO donors might serve as therapeutic alternatives in cancer treatment because of their anti-proliferative and cytotoxic activities [160]. High NO/SNO concentrations activate the intrinsic apoptotic cell death pathway, leading to killing of neurons, macrophages and tumor cells [163–167]. Elevated NO concentrations may also activate other signaling pathways that control tumor progression [168].

The use of NO donors as potential chemotherapeutic agents has been increasingly described. Studies performed *in vitro* with cancer cell lines, *in vivo,* with implanted cancer cell lines in immunosuppressed mice and *in vivo,* in clinical trials involving the use of NO donors in patients with chemotherapeutic resistant cancers have been conducted resulting in promising outcomes [160].

5.2.1. In vitro studies

The NO donor, *S*-nitrosocysteine ethyl esther inhibits the activity of the protein phosphatase Cdc25 A in the HCT116 colon cancer cell line [169]. Cdc25 A is over-expressed in various human cancers and is a suppressor of apoptosis [170].

Glyceryl trinitrate (GTN) at millimolar concentrations induces apoptotic cell death in SW480 human colon cancer cell lines [171]. The NO donor DETA – NONOate inhibits the activity of the metastasis inducers NFκB and Snail**,** and stimulates the metastasis suppressor protein, Raf kinase inhibitor protein - RKiP [172].

DETA-NONOate inhibits the EMT in human prostate cancer cell lines [170]. Similarly, millimolar concentrations of the s-nitrosothiol SNAP, inhibits the TGF-β-induced EMT in mouse hepatocytes [173].

Increasing NO concentrations may cause changes in the extracellular matrix that lead to tumor cell death. Spermine-NONOate at concentrations ranging from 10 μM to 1 mM promotes *S*-nitrosylation of key cysteine residues in the matrix metalloproteinase 9 (MMP-9), stimulating its activity [174]. MMP-9 activation may lead to extracellular matrix degradation, resulting in cell death by anoikis triggered by inadequate or inappropriate contact between the cell and the extracellular matrix [175].

NO has anti-adhesive properties that inhibit cell adhesion to a substrate, affecting the organization of focal adhesion complexes and inhibiting cell-extracellular matrix interactions. Based on these anti-adhesive activities, high NO concentrations are potential mediators of anoikis [175]. The anti-adhesive and pro-apoptotic activities of NO have been targeted in A431 human vulvar carcinoma cell lines that do not express PTPα. When the major constituents of the focal adhesion complex, particularly FAK, Src kinase, and PTPα are expressed in these cancer cells, NO-mediated cell death occurs without detachment from the substrate, showing characteristics of necrosis [176].

5.2.2. In vivo pre-clinical studies

Oral administration of GSNO, an NO donor of the *S*-nitrosothiol class, to immunosuppressed mice implanted with A2780 human ovarian cancer cells has resulted in a significant decrease in the tumor mass located in the peritoneum [177].

Furoxans, molecules bearing 1,2-5-oxadiazole *N*-oxide moiety are NO donors that possess anticancer activity by interfering with *in vivo* NO concentrations via a thiol-dependent NO release mechanism [178].

Phenylsulfonylfuroxan-anilinopyrimidine hybrid compounds inhibit the growth of tumors derived from H1975 human non-small cell lung cancer cells implanted in athymic BALB/c mice [179], while phenylsulfonylfuroxan-based hydroxamates which are NO donors and histone deacetylase inhibitors, induce apoptosis and G1 phase arrest in human erythroleukemia cells [180].

5.2.3. In vivo clinical studies

In a Phase I clinical trial of patients with colon cancer, transdermal patches containing GTN and 5-fluorouracil caused mild to moderate toxicities [181]. A survey of preclinical studies and clinical trials using GTN alone or combined with chemotherapeutic agents to treat prostate, lung and colon cancers indicates that the use of GTN may be beneficial to patients and that GTN combinations with other chemotherapeutic agents show promise [182].

GTN has also been used in a Phase II clinical trial as a chemosensitising agent in patients with stage IIIb/IV non-small cell lung cancer treated in combination with vinorelbine and cisplatin. Improvement has been observed in patient response to the chemotherapeutic agents accompanied by a delay in disease progression [183]. In a second Phase II clinical trial, GTN administered to 29 patients with recurring prostate cancer non-responsive to primary treatment has caused deceleration and/or stabilization of the prostate-specific antigen (PSA) levels [184].

In a Phase I clinical trial, 25 patients with incurable solid tumors have received increasing doses of the NO and ROS donor, 1-bromoacetyl-3, 3-dinitroazetidine (RRx-001). Four patients became sensitive again to a treatment that they had become resistant to before exposure to RRx-001 [185]. RRx-001 functions as a platinum-based chemotherapy sensitizer in refractory small-cell lung carcinoma [186] and a radio sensitizer in brain metastases and glioblastoma [187]. In squamous cell carcinoma SCC VII cells, RRx-001 decreases global DNA methylation and increases global acetylated histones H3 and H4 [188].

5.3. NO production by immune cells of the tumor microenvironment and immunogenic cell death

The tumor microenvironment consists of non-cancerous cells present in the tumor, including endothelial cells, fibroblasts, and cells from the immune system [2]. Understanding cellular communication between immune and cancer cells is challenging, but it may explain how this interaction affects tumor development. The role of NO in the tumor microenvironment depends on its local concentration where it may contribute to tumor growth or elimination [134,135]. In the microenvironment NO may be derived from cancer cells and from cells of the innate immune system, acting on both cell populations. Cells of the innate immune system, such as macrophages, dendritic cells (DCs) and natural killers, can be activated in the presence of NO and promote tumor elimination [189].

Cancer cells in general are not immunogenic, and a protective specific anti-tumor immune response is not detected during tumor development. However, in specific situations this condition can revert leading to immunogenic cell death (ICD).

The occurrence of ICD was first proposed in the context of the use of some conventional chemotherapy agents. It was based on clinical evidence of tumor-specific immune responses detected during these therapies, which explains the increased efficacy of these selected conventional chemotherapeutic agents [190]. Immunocompetent individuals respond better to these chemotherapeutic agents than immunodeficient individuals [191].

Evaluation of the capacity of novel chemotherapeutic candidates to induce ICD has been encouraging, as demonstrated by the ability of R2016, a heterocyclic quinone derivative, to induce ICD in LLC murine lung cancer cells and in B16F10 murine melanoma cells [192]. ICD induction also occurs with anthracyclines, daunorubicin, doxorubicin, docetaxel, mitoxantrone, paclitaxel and oxaliplatin [193].

ICD involves cancer cell expression and release of specific proteins termed damage-associated molecular patterns (DAMPs). DAMPs that are involved in ICD include calreticulin and other endoplasmic reticulum proteins, which are expressed on the cell surface. These molecules are recognized by receptors expressed by DCs and induce the presentation of tumor antigens to T lymphocytes [194]. DC receptors involved in ICD induction that recognize DAMPs released by cancer cells undergoing the immunogenic death process, include the Toll-like receptor 4 and the P2X7 purinergic receptor [194].

The important role played by NO during ICD induction in cancer cells has been demonstrated. The dependence of anthracycline-induced calreticulin exposure on endoplasmic reticulum stress involves the formation of ROS and NO derived from NOS2 activation in cancer cells [189,195]. Doxorubicin promotes NOS2 activation through NF-kB activation in HT29 human colon carcinoma cells [189]. The effect of doxorubicin on NF-kB and NOS2 expression increases NO production leading to calreticulin exposure by cancer cells [196]. In NOS2-knockout HT29 cells, the NO donor SNP re-establishes the expression of calreticulin on the cell surface [189]. This is another property that may be exploited in chemotherapeutic regimens based on the use of NO donors.

DCs activated by ICD-induced cancer cells may produce different concentrations of NO, and this effect can influence the efficiency of the induced immune response. A novel ruthenium-derived chemotherapeutic candidate (Ru) induces ICD in 4T1 murine breast adenocarcinoma, exerting a protective anti-tumor immune response *in vivo*, although less efficiently than oxaliplatin. DCs activated *in vitro* with Ru treated 4T1 cells produce higher concentrations of NO as compared to oxaliplatin treated 4T1 cells, suggesting that NO generated by activated DCs can negatively modulate the *in vivo* induced immune response [197].

6. Conclusions

The evidence summarized in this review leads to the following hypothesis which explains the dynamics of tumor progression from a redox signaling perspective: cancer cells activate different processes to control NO and ROS concentrations at the different stages of tumor development, with lower concentrations of NO and higher concentrations of ROS at the initial phases of development. The situation reverses when the levels of these species are determined in tumors that have undergone the EMT. NO levels rise to activate the oncogenic signaling pathways that positively regulate the EMT. Further increases in NO concentrations are necessary for providing the circulating cancer cells with resistance to anoikis. When cancer cells undergo the MET and successfully established themselves at the metastatic site, a return to the condition of lower levels of NO and higher levels of ROS has to occur. This hypothesis is graphically described in Fig. 5.

Therefore, a fine spatial-temporal control of ROS and NO concentrations must be operational for the successful completion of all tumor stages. By decreasing and/or increasing the levels of these species “at the wrong time” during tumor progression, the process can be seriously disturbed, opening new possibilities for redox–based therapeutic interventions.

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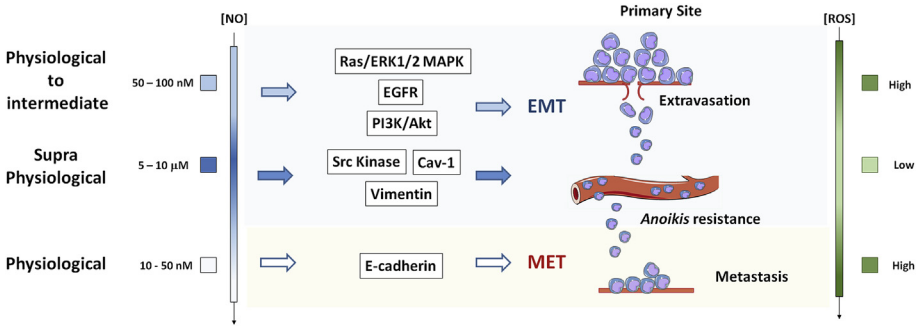

Fig. 5. Production of NO and ROS at different stages of tumor development. In the initial stages of tumor development, ROS prevail whereas the cancer cells negatively regulate endogenous production of NO. Induction of **the** EMT is accompanied by elevation of NO levels in the estimated concentration range of 50–100 nM, defined as physiological to intermediate levels. Further increase in NO concentrations is necessary for cancer cells to extravasate, invade lymph nodes and adjacent tissues, and then migrate through the blood stream, acquiring anoikis resistance. Estimated levels of NO in this situation are within the range of 5–10 µM, defined as supra physiological concentrations. After extravasation and re-adhesion of migrating cancer cells with their establishment at the site of metastasis, the role of ROS should prevail with a concomitant decrease in NO concentrations. Estimated concentrations of NO in this situation are within the range of 10–50 nM, defined as physiological levels. Estimation of NO concentrations was based on the information discussed in Refs. [26,28,117,118]. (Figure was elaborated using the source: Servier Medical Art. https://smart.servier.com).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.04.009. 501100003593

Conflicts of interest

The authors declare no conflict of interest.

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