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S-nitrosylation and its role in breast cancer angiogenesis and metastasis

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A B S T R A C T

S-nitrosylation, the modification by nitric oxide of free sulfhydryl groups in cysteines, has become an important regulatory mechanism in carcinogenesis and metastasis. S-nitrosylation of targets in tumor cells contributes to metastasis regulating epithelial to mesenchymal transition, migration and invasion. In the tumor environment, the role of S-nitrosylation in endothelium has not been addressed; however, the evidence points out that S-nitrosylation of endothelial proteins may regulate angiogenesis, adhesion of tumor cells to the endothelium, intra and extravasation of tumor cells and contribute to metastasis.

1. Introduction

S-nitrosylation is an important posttranslational modification induced by nitric oxide (NO) that depends on the local concentration of NO and affects intracellular traffic process, protein phosphorylation and protein-protein interactions [1–3]. S-nitrosylation consists in the coupling of an NO moiety to a reactive cysteine thiol to form an S-nitrosothiol [4,5]. This modification is independent of the soluble guanylate cyclase and protein kinase G (sGC-PKG) pathway classically activated by NO. S-nitrosylation is regulated negatively by two enzyme systems that mediate denitrosylation: S-nitrosoglutathione reductase (GSNO-R) and thioredoxin [6]. The inhibition or malfunction of any of these systems leads to high levels of S-nitrosylated proteins. S-nitrosylation depends on NO induced by different isoforms of nitric oxide synthase: eNOS (endothelial), iNOS (inducible) and nNOS (neuronal) [7]. eNOS and nNOS induce NO concentrations in the nanomolar range whereas iNOS induce NO concentrations in the micromolar range [9]. It is considered that NO concentrations above 100 nM induce S-nitrosylation. These concentrations can be easily reached by iNOS and also by eNOS when this is activated [8,9]. Protein S-nitrosylation is usually measured by the biotin switch method where S-nitrosylated cysteines are converted to biotinylated cysteines. After further purification by biotin-affinity isolation, the degree of biotinylation (and hence S-nitrosylation) is determined by either anti-biotin Western-blot or streptavidin pull down followed by immunoblotting for the protein (s) of interest [10]. S-Nitrosylation has been implicated in cardiovascular, pulmonary, musculoskeletal and neurological dysfunction, including cancer [11–13]. Accumulating evidence suggests that NO production and dysregulated S-nitrosylation are a key event that increases cancer risk and metastasis [14-16]. In this review we will focus in breast cancer and how S-nitrosylation of proteins in endothelial and tumor cells contributes to metastasis with special emphasis on S-nitrosylation of adherens junction proteins.

2. Breast cancer and S-nitrosylation

Breast cancer is the most commonly diagnosed cancer and the leading cause of death from cancer in women [17,18] (https://www.cancer.gov/types/breast). It is estimated that 18.1 million new cases of cancer and 9.6 million deaths from cancer will be diagnosed this year 2018. Different subtypes of breast cancer are distinguished according to the presence of estrogen (ER) and progesterone (PR) receptor, as well as epidermal growth factor receptor (HER-2): a) ER/PR positive (luminal A/B) and b) triple negative (ER, PR, and HER-2 negative) (basal-like) (TNBC) [19]. Patients with triple negative breast cancer present the more malignant phenotype [20].

High levels of NO have been detected in blood of breast cancer patients and increased NOS activity (eNOS, iNOS) has been detected in invasive breast tumors compared to benign or normal breast tissue, independently of ER expression, suggesting a positive correlation between NO biosynthesis and degree of malignancy [21–26]. Tumor cells express iNOS, eNOS and nNOS, depending on tumor type and stage. Tumor endothelial cells express eNOS and iNOS whereas tumor-associated stromal fibroblasts and immune cells express iNOS [27].

S-nitrosylation mediated by eNOS and iNOS regulate activation of oncogenic signaling pathways, loss of tumor suppressor activity, transcription of genes involved in metastasis, EGF-R activation and invasion of tumor cells [28–31]. Furthermore GSNO-R downregulation and

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Table 1
S-nitrosylated proteins in endothelial cells.

Pathways Involved	Identified proteins	Model	Reference
Cytoskeleton regulation, metabolism.	Cytoskeletal proteins, β-actin, γ-actin, vimentin, GAPDH, tropomyosin.	EAhy926 cells treated with NO donors. Human aortic endothelial cells (HAEC) treated with NO donors.	[34–36]
Apoptosis, cell structure and metabolism, redox homeostasis viability, cell cycle, signaling, gene expression, DNA replication/recombination/repair, energy production, molecular transport, migration/morphology, protein synthesis, cardiovascular system development and function, antigen presentation.	GAPDH, enolase1, phosphofructokinase, peroxiredoxin 2, eukaryotic translation initiation factor 5A.	HUVEC treated with estrogen.	[37]
Metabolism and molecular transport, cellular assembly and organization, signaling, protein modification, cell viability and proliferation, cell cycle, cellular movement, DNA replication/recombination/repair, gene expression, cytoskeleton regulation, cell adhesion, cell death.	Aconitase II, pyruvate kinase, zyxin, vimentin, spectrin, fascin, cofilin-1.	Uterine artery endothelial cells (UAEC) treated with estradiol-17β.	[38]
Endothelial cell proliferation, cell cycle, cytoskeleton and motility, metabolism, protein synthase and modification, cellular signaling and transportation.	Calpain-2 catalytic subunit, calpain small subunit 1, galectin-1, poly (rC)- binding protein 2, GTP-binding nuclear protein Ran, protein S100-A11, interleukin enhancer-binding factor 3, DNA replication licensing factor MCM5, and four 14-3-3 proteins, cofilin-1, myosin, fasin, and actin-related protein 2/3 complex (ARP2/3), EIF2.	Primary placental endothelial cells treated with NO donors and vascular endothelial growth factor (VEGFA).	[39]
Ribosomal structure, translational regulation, apoptosis and cell adhesion.	EEF2, RPS3, α-catenin, p120, β-catenin, N-cadherin, VE-cadherin, VASP.	EAhy926 cells treated with oxidized-LDL	[40]

therefore increased S-nitrosylation are associated with drug resistance and poor prognosis in breast cancer [32,33].

3. Endothelial function modulated by S-nitrosylation and association to metastasis

Large scale analysis of endothelial S-nitrosylated proteins has led to the identification of about 400 proteins [34–39]. The studies used different endothelial cells such as HUVEC, EAhy926 cells, pancreatic mouse endothelial cells, uterine arterial endothelial cells and ovine fetoplacental artery endothelial cells (oFPAEC) incubated with NO donors and physiological stimuli (estrogen, VEGF) [34–39]. Many of the identified proteins (Table 1) were classified as participating in pathways related to cytoskeleton and motility. Targets involved in cell adhesion and adherens junction signaling were also identified [37–39].

Some of this proteomic analysis have suggested that S-nitrosylation of endothelial proteins could be involved in angiogenesis [39] pregnancy-associated uterine vasodilatation which is directly linked to fetal growth [38] and atherosclerosis [40]. Future work on S-nitrosoproteome analysis of clinic samples will help to understand the role of S-nitrosylation in different diseases where endothelial dysfunction is key. Rearding to that, in pre-eclampsia, a condition where endothelial function is impaired, the identification of the S-nitrosoproteoma derived from clinical samples showed strong differences between pre-eclampsia and normal placentas suggesting that this modification is implicated in this pathology [41].

During metastasis, endothelial cells play a key role (Fig. 1). They allow tumor growth through angiogenesis, they modulate the entry of tumor cells to the circulation and their exit in the metastatic site. Despite advances in the knowledge of S-nitrosylated proteins in endothelial cells, proteomic approaches have not assessed the pathways

Fig. 1. Metastatic cascade. 1) Angiogenesis and separation of tumor cells from the primary tumor and migration into the adjacent tissue; 2) invasion of tumoral cells through the tissues, 3) intravasation of tumoral cells into blood and/or lymphatic circulation; 4) survival of the cells in the circulation (anoikis resistance); 5) extravasation of the cells out of the circulation and 6) colonization, proliferation, and angiogenesis of tumor cells at the distant sites to form a secondary tumor.

involved in metastasis. Many of the identified proteins were classified as participating in pathways related to cytoskeleton and motility that may participate in endothelial migration and angiogenesis. Targets involved in cell adhesion and adherens junction signaling may be also involved in angiogenesis and endothelial permeability during metastasis. Nonetheless, the endothelial S-nitrosoproteome that develops in cancer has not been systematically investigated. In this section we revise the endothelial proteins that are S-nitrosylated and may modulate endothelial function associated to metastasis.

S-nitrosylation Modulates Endothelial Permeability: Tumors display a progressive instability of the endothelial barrier leading to hyperpermeability, which can correlate with tumor grade [42]. Endothelial hyperpermeability contributes to generate an environment enriched in plasma proteins, including a fibrin-enriched stroma crucial for tumoral growth and angiogenesis [42]. It also facilitates the entry of neoplastic cells into blood vessels (intravasation) and their exit in targets organs (extravasation) during metastasis [43,44].

The stability of the endothelial barrier is maintained mainly by the adherens junction formed by VE-cadherin in a complex with p120, β-catenin, α-catenin and γ-catenin [45]. This structure is present in all vasculatures and controls the passage of tumoral cells to tissues [46,47]. A strong correlation exists between destabilization of the adherens junction, endothelial permeability and metastasis [46–48].

We have demonstrated the key role of eNOS in endothelial permeability using eNOS knockout mice and eNOS depleted endothelial cells [49,50]. Because of potency and broad spectrum of the cellular actions of NO and its brief half-life, the mechanisms that regulate NO synthesis with respect to time and space are crucial in determining the biological functions of NO. In the plasma membrane, eNOS localizes preferentially to caveolae and interacts with caveolin-1, which keeps eNOS activity at basal levels [51]. We demonstrated that upon agonist stimulation, eNOS is activated by phosphorylation, released from the inhibitory interaction with caveolin-1, and internalized through caveolar endocytosis to promote endothelial permeability [50,52,53]. Using eNOS mutants specifically located to the cytosol or plasma membrane, we demonstrated the fundamental role of eNOS localization at the cytosol for the onset of the increase in permeability [8]. Importantly, we demonstrated that S-nitrosylation of adherens junction proteins, rather than PKG-cGMP pathway, determines the increase in permeability. S-nitrosylation of p120, β-catenin and VE-cadherin leads to changes in the protein-protein interactions at the adherens junction resulting in internalization of junctional proteins, and destabilization of the endothelial barrier, which results in increased microvascular permeability [2,3]. β-catenin is also S-nitrosylated in Cys466 in endothelial cells and this S-nitrosylation inhibits the β-catenin transcriptional activity [54].

At the molecular level, Cys619 was identified as the S-nitrosylated residue in β-catenin [55]. For p120, we identified 5 S-nitrosylated cysteines in response to NO donor [2]. Three of them, Cys429, Cys450 and Cys579 were located in the molecular stretch of amino acids 352-612 that interacts with E-cadherin [56–58] and by sequence homology may also interact with VE-cadherin. S-nitrosylation of these cysteines might represent a critical event associated with disruption of the endothelial barrier. In regards to VE-cadherin, we demonstrated that inhibition of S-nitrosylation by L-NMA or NAC treatment blocks VE-cadherin phosphorylation and internalization [3]. S-nitrosylation of VE-cadherin may promote a conformational change in VE-cadherin that induces its phosphorylation. VE-cadherin has one cysteine (Cys607) in the transmembrane domain very close to the intracellular domain and four cysteines in the extracellular domain. Since permeability and S-nitrosylation of adherens junction proteins depend on eNOS cytosolic localization [8], Cys607 could be the cysteine that is S-nitrosylated. It is plausible that in the tumoral microenvironment, where high NO concentrations may be reached due to NO being produced by tumor, immune and stromal cells, the extracellular cysteines in VE-cadherin may also be S-nitrosylated. This could cause conformational changes that disrupt the homotypic interaction of VE-cadherin in adjacent cells. In fact, four extracellular cysteines are located in the EC5 domain of VE-cadherin that participates in the homotypic adhesion [59].

The stability of the endothelial barrier also depends on the actin–myosin contractility and adhesiveness of integrins to focal adhesions [60,61]. Focal adhesions are contact points of integrins with matrix ligands that provide anchorage to the extracellular matrix. When endothelial permeability increases, focal adhesions are reorganized to stabilize the lateral contractile forces during the increase in permeability. On the other hand, focal adhesion may actively contribute to endothelial permeability providing a mechanical basis for cells to contract [62]. Vasodilator-stimulated phosphoprotein (VASP) belongs to the Ena/VASP family of actin regulatory proteins and is a main element of focal adhesions [63]. The key role of VASP in maintaining barrier function is supported by evidence showing that mice lacking proteins of the VASP family die from edema due to defective vascular barrier function [64] and that VASP-deficient endothelial cells show increased permeability under basal conditions [63,65]. Using VASP KO endothelial cells transfected with different VASP cysteines mutants, we demonstrated that S-nitrosylation of VASP at Cys64 promotes endothelial permeability [66]. Cys64 is located in the EVH1 domain of VASP, which mediates the subcellular targeting of VASP to focal adhesions through interactions with zyxin and vinculin [67,68]. Therefore, S-nitrosylation of VASP may regulate its localization and interactions with other proteins to promote reorganization of focal adhesions and increase endothelial permeability. A summary of the role of S-nitrosylation in endothelial permeability is shown in Fig. 2.

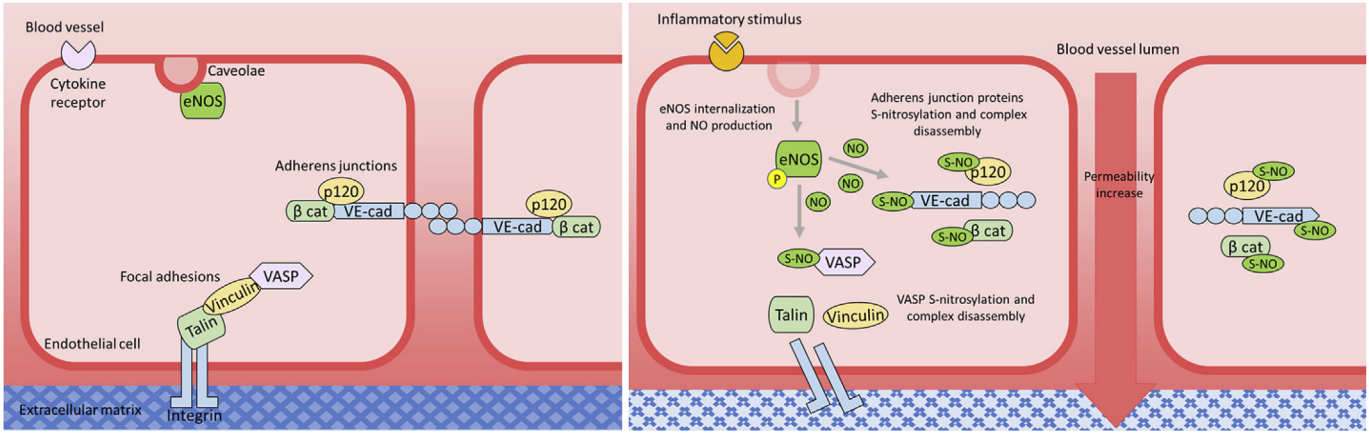

Fig. 2. Regulation of endothelial permeability by S-nitrosylation. In response to an inflammatory stimulus, eNOS is internalized and S-nitrosylates adherens junction proteins and VASP to promote disassembly and internalization of adherens junction proteins and VASP re-organization at focal adhesions.

S-nitrosylation of targets involved in angiogenesis: Angiogenesis, the process by which endothelial cells proliferate and reorganize to form new blood vessels, is key for tumor growth and metastasis [70]. Hypoxia-inducible factor 1α (HIF-1α) is a constituent of the transcription factor HIF-1 and it is up-regulated in hypoxic condition. In the tumor microenvironment, HIF-1α promotes metastasis of breast cancer cells [71]. In breast cancer cells, HIF-1α is S-nitrosylated at Cys533 in normoxic conditions stabilizing the protein and up-regulating its activity, which leads to increased expression of angiogenic factors that act on endothelial cells promoting angiogenesis [72].

Other S-nitrosylated proteins involved in angiogenesis in another contexts have been identified but its role in tumor angiogenesis requires further investigation:

MAPK phosphatase 7 (MKP-7): It is part of the signaling cascade stimulated by stromal cell-derived factor-1α (SDF-1α) to promote angiogenesis and endothelial cell migration. SDF-1α induced activation of JNK3 is critical for endothelial cell migration and depends on eNOS activity. eNOS induces S-nitrosylation of MKP-7, lowering its phosphatase activity and in this way is unable to inhibit the activation of JNK3, which enhance endothelial migration [73]. Future experiments are needed to determine whether S-nitrosylation of MKP-7 contribute to tumor angiogenesis.

cofillin-1: Is a small actin-binding protein, essential for actin cytoskeleton remodeling. S-nitrosylation of cofillin-1 in response to VEGF and GSNO stimulates endothelial migration which can lead to angiogenesis important in metastasis. Cys80 and Cys139 were identified as the major SNO-sites in CFL1 by LC-MS/MS [74]. The significance of cofillin-1 S-nitrosylation in tumor angiogenesis needs to be investigated.

Cyclooxygenase-2 (COX-2): COX-2 overexpression has been linked with tumor progression, invasion and metastasis in breast cancer [75]. COX-2 stimulates angiogenesis through the expression of angiogenic factors such as VEGF in the tumor cell that act in a paracrine way on endothelial cells to promote angiogenesis [75] In other cellular models (heart and neurons) it has been demonstrated that NO activates COX-2 by S-nitrosylation [76,77]. It is unknown if the same mechanisms may activate COX-2 in the context of tumor angiogenesis.

Epidermal growth factor receptor (EGF-R): The treatment of endothelial cells with bradykinin induces angiogenesis by stimulation of EGF-R kinase activity through S-nitrosylation [78] and S-nitrosylation of EGF-R regulates its kinase activity [79]. Since endothelial cells from tumors express EGF-R [80] it would be interesting to investigate if S-nitrosylation of EGF-R in this context regulates tumor angiogenesis.

Transient receptor potential channels (TRPs): They are a group of ion channels located mainly on the plasma membrane of numerous animal cell types. In addition to its role in the nervous system, TRPs are overexpressed in breast cancer and regulate migration, invasion, EMT, extravasation, angiogenesis and metastasis [81,82]. Cys553 and 558 were identified as cysteines S-nitrosylated in TRPC5, which activates the channel [83]. Alignment sequence showed that these cysteines are conserved in TRPC4, which is crucial for endothelial cell migration in breast tumors [82].

S-nitrosylation of endothelial targets involved in tumor cell adhesion and transmigration: Once tumor cells have reached the vascular system, only a small proportion may be viable and have the potential to extravasate [84]. The role of NO in adhesion and transmigration of tumor cells is controversial. NO donors and endogenous NO enhanced fibrosarcome cell adhesion invasion through the HUVEC monolayer increasing ELAM expression [85]. *In vivo*, breast circulating tumoral cells MDA-MB-231 adhered to microvessel sites with high NO concentrations generated by shear gradients induced by blood flow, while eNOS inhibition in this system reduced tumor cell adhesion [86]. On the contrary, NO inhibited the adhesion of colorectal cancer cells to the endothelium [87]. COX-2 and HIF-1α expressed in endothelial cells are involved in extravasation of breast cancer cells through the expression of MMP-2 and regulation of the expression of adhesion molecules that bind to tumor cells, respectively [88,89]. Both proteins are activated by S-nitrosylation in others cellular contexts [72,75,77]. Whether or not S-nitrosylation activates these proteins in endothelial cells remains an open question.

Another target that may be involved in tumor cell adhesion is PKCζ. Overexpression of PKCζ is associated with advanced breast cancer, lymph node metastasis, and poor survival rates [90]. Interestingly, a PKCζ synthetic peptide containing the activation site is S-nitrosylated *in vitro* in cysteine 412 [91,92]; however, the effect of the modification on PKCζ activity is unknown, but it may activate the protein since a study showed that the inhibitor of S-nitrosylation N-ethylmaleimide decreases the activity of PKC, suggesting the functional role of the thiol groups in this molecule [93]. In endothelial cells, PKCζ activates ICAM-1 phosphorylation and clustering that promotes leukocyte adhesion [94,95]. Tumor cells in the blood stream can bind to circulating monocytes/macrophages and leukocytes; these cells bind to the endothelium through adhesion proteins acting as a bridge between the tumor cell and the endothelium promoting cancer cell extravasation into surrounding tissues [43]. ICAM-1 expression in the endothelium is critical to bind these intermediary cells [43,96]. We are currently testing the role of PKCζ − S-nitrosylation in tumor cell adhesion.

4. S-nitrosylation of tumor targets and its role in breast cancer metastasis

In recent years the number of S-nitrosylated targets has increased significantly with the last update in 2015 showing 2277 S-nitrosylated proteins in different organisms [97] (http://dbSNO.mbc.nctu.edu.tw/). Analysis of the dbSNO data base showed that 51% of 720 human S-nitrosylated proteins matched to cancers, including breast cancer [97]. In addition, specific tumoral S-nitrosoproteome has been identified in colorectal carcinoma [98] and lung cancer cells [99]. However, the identification of the SNO-proteome that develops in breast cancer has not been addressed yet. Here, we review S-nitrosylated proteins that promote metastasis affecting tumor cell function.

The composition of the tumoral adherens junction is similar to the endothelial adherens junction, but instead of VE-cadherin, E-cadherin forms the complex with α-, β-, γ- and p120 catenins which link E-cadherin to actin cytoskeleton [100]. A hallmark of cancer progression is epithelial-to mesenchymal transition (EMT) [101]. During this process E-cadherin is downregulated and the cells gain a mesenchymal, migratory and invasive phenotype. In breast cancer there is a significant association between reduced or abnormal (non-membrane and cytosolic) expressions of adherens junction proteins and metastasis [102–104].

Several reports support a role for S-nitrosylation in the integrity of the tumoral adherens junction, EMT and metastasis. Long-term treatment of non-small lung cancer cells with NO donors significantly decreased E-cadherin levels at the same time that increased vimentin and snail levels [105]. *S*-nitrosylation of Src at cysteine 498 in breast cancer MCF-7 cells promotes Src activation leading to disruption of the epithelial adherens junctions by reducing the expression of E-cadherin at the plasma membrane, which enhances cell invasion [26]. *S*-nitrosylation of Src in a more invasive breast cancer cell line, MDA-MB-468, also correlates with Src activation, decreased expression of E-cadherin and increased expression of EMT markers [29]. S-nitrosylation of EGF-R-in MDA-MB-468 breast cancer cells leads to activation of EGF-R, β-catenin signaling and EMT [29]. It is, thus, plausible that high level of NOS activity in the tumor may induce the disruption of adherens junction in tumoral cells.

S-nitrosylation also may affect tumoral adherens junctions in a manner similar to its effect on endothelial adherens junctions, and lead to metastasis by further altering barrier function. Indeed, α-catenin is S-nitrosylated in epithelial prostate cells [100]. While the function of S-nitrosylated α-catenin is unknown, it may affect its interaction with other

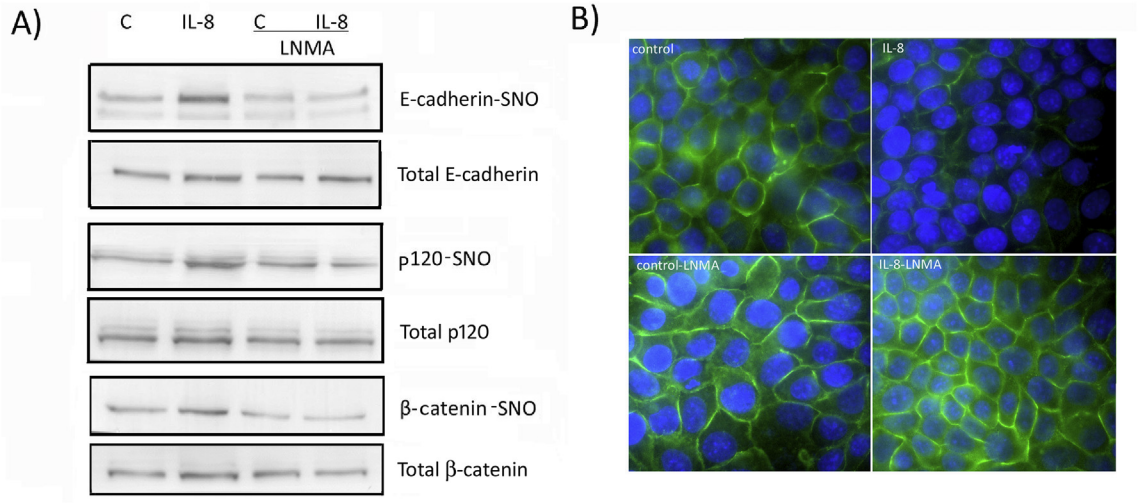

Fig. 3. S-nitrosylation of adherens junction proteins in tumor cells. A) MCF-7 cells were stimulated with 100 nM IL-8 72h in presence or absence of 300 μM L-NMA and processed by biotin-switch assay. B) MCF-7 cells were stimulated with 100 nM IL-8 72h in presence or absence of 300 μM L-NMA. E-cadherin was observed by indirect immunofluorescence.

proteins and cause disruption of the adherens junction. Our preliminary data (Fig. 3) show that IL-8 induces S-nitrosylation of E-cadherin, p120 and β-catenin in MCF-7 cells. S-nitrosylation correlated with diminished levels of E-cadherin at the plasma membrane. These results suggest that S-nitrosylation may dysregulate the tumoral adherens junction and cause enhanced migration of these cells.

Other S-nitrosylated proteins that mediate metastasis in breast cancer are Ras and HIF-1α. Ras S-nitrosylation activated Ets-1 transcriptional activity in breast cancer cells via phosphorylation through the Ras/MEK/ERK pathway. Ets-1 is a transcription factor that can promote an aggressive cancer cell phenotype involved in the progression of basal-type breast cancer and related to a poor survival of the disease. Activation of Ets-1 resulted in increased expression and activity of MMPs increasing cancer cell invasion and proliferation [31]. HIF-1α regulates expression of EMT markers, cell migration as well as angiogenic factors to promote metastasis of breast cancer cells [71]. HIF-1α is S-nitrosylated at Cys533 in normoxic conditions in breast cancer cells stabilizing the protein and up-regulating its activity [72].

Proteomic analysis has demonstrated that other proteins are also S-nitrosylated and may modulate metastasis; however, their role in breast cancer metastasis has not been addressed yet:

VASP: Increased expression levels of VASP have been associated with breast cancer metastasis by positively regulating migration and invasion of breast cancer cells [106,107]. VASP was identified as a target S-nitrosylated in a proteomic analysis of prostate epithelial cells [108]. In endothelial cells, we identified Cys64 in VASP as the cysteine responsible for the increase in endothelial permeability [66]. As we mention before, Cys64 mediate interaction with zyxin and vinculin in the focal adhesions [67,68]. It will be interesting to test whether S-nitrosylation of VASP can regulate migration and invasion of tumor cells.

Metalloproteinases (MMPs). They are extracellular matrix-degrading endopeptidases that facilitates cancer cell invasion [69]. In breast cancer, MMP-9 expression is associated with cell invasion [110]. MMP-9 has been reported to be activated by S-nitrosylation in the central nervous system and trophoblasts leading to neuronal apoptosis and increased trophoblast migration and invasion [111], respectively. Thus, it is possible that MMP-9 S-nitrosylation in tumors may promote metastasis improving tumor cell invasion.

Cyclooxygenase-2 (COX-2): In breast cancer an strong correlation between COX-2 expression and metastasis have been demonstrated [112,113]. As we mention before NO activates COX-2 by S-nitrosylation in hearth and neurons [76,77]. Whether this modification takes place in breast cancer cells is unknown.

α6-integrin: Integrin α6 is a laminin adhesion receptor with an established role in invasion and migration of breast cancer cells [114]. S-nitrosylation of integrin α6 at Cys86 by iNOS in prostate cancer, increases the extent of prostate cancer cell migration by enhancing ITGα6-ITGβ1 heterodimerization and loss of ITGα6 binding to laminin-β1 [115]. It would be interesting investigate if in breast cancer cells this integrin is regulated by S-nitrosylation and in this way modulate cell migration.

Caveolin-1: It is an integral membrane protein involved in caveolae biogenesis, cholesterol homeostasis, intracellular trafficking and signal transduction [116]. In breast cancer, caveolin-1 expression is known to be up-regulated and protein levels correlate with tumor progression and metastasis [117]. *S*-nitrosylation of caveolin-1 inhibits its ubiquitination and proteasomal degradation improving anoikis resistance in lung carcinoma cells [105]. In breast cancer cells caveolin-1 also prevent anoikis [118] and could be regulated by S-nitrosylation in the same way than in lung cancer cells.

NFKB: NFKB activity is upregulated in many cancers and promotes the expression of many genes involved in survival, proliferation, invasion and metastasis of tumor cells [119,120]. NFKB can also either inhibit or promote apoptosis [120]. Constitutive activation of NFKB has been detected in breast cancer and NFKB inhibition in breast cancer cells promotes apoptosis [121]. S-nitrosylation is considered an important mechanism of inhibition of NF-kB signaling [119]. Under physiological conditions, NFKB is kept in an inactivated state by constitutive S-nitrosylation of IKKβ. After TNFα stimulation, IKKB is denitrosylated and NFKB is activated. Furthermore, NFKB subunits, p50 and p65 are also S-nitrosylated and the modification inhibits the binding of NFKB to the DNA. Thus S-nitrosylation negatively regulates NF-kB activity [119]. However some evidence has shown a positive regulation of NFKB activity by S-nitrosylation. In this context NFKB signaling promotes apoptosis [122,123].

Apoptosis proteins: S-nitrosylation regulates several proteins in apoptotic pathways. Caspases for example can be activated or inhibited by S-nitrosylation [119]. S-nitrosylation of the death receptor DR4 and FAS promotes apoptosis [119,124,125]. S-nitrosylation of Ying Yang 1, an inhibitor of FAS, blocks its inhibitory activity and allows dowstream apoptotic signal transduction. On the other hand, the S-nitrosylation of cFLIP, an inhibitor of apoptotic pathways, blocks its degradation making it a more potent inhibitor of apoptosis. Furthermore, S-nitrosylated cFLIP activate the NF-KB signaling pathway leading to cell proliferation and survival [125]. Interestingly, activation and S-

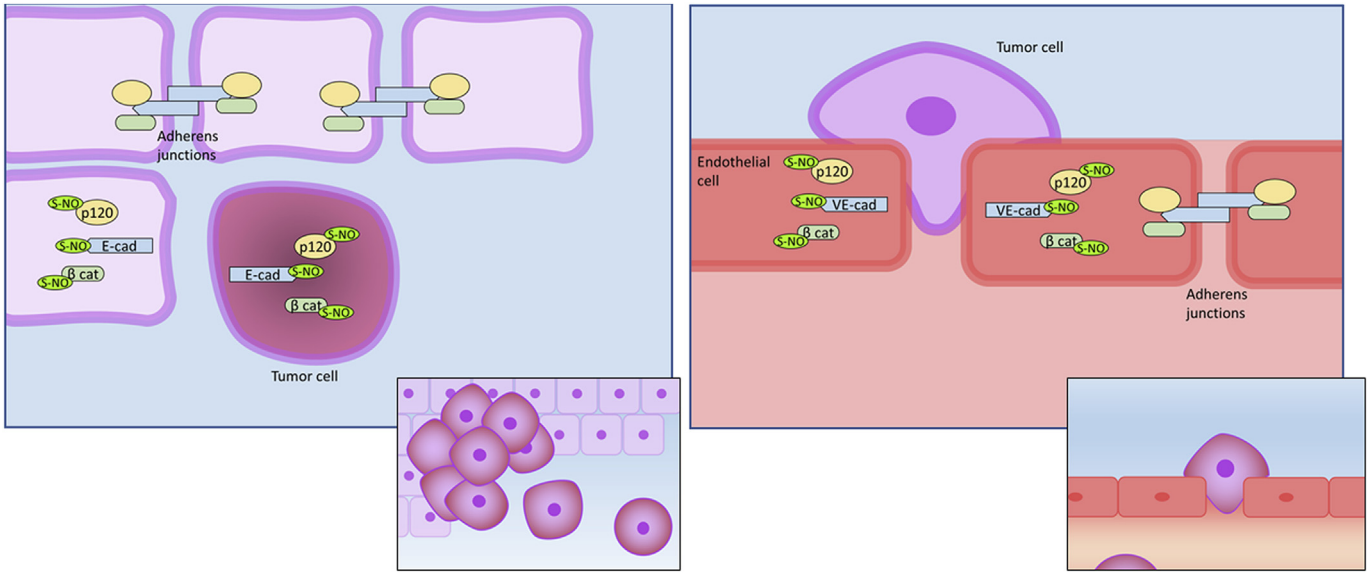

Fig. 4. Proposed model of mechanisms through which S-nitrosylation of adherens junction complex increases tumoral metastasis. S-nitrosylation of adherens junction proteins facilitates EMT-migration in tumoral cells and the disruption of the endothelial barrier promotes intra and extravasation of tumor cells.

nitrosylation of Src has been correlated with anoikis resistance in HeLa cells and melanoma cells [126]. Anoikis has been described as an important mechanism involved in the metastasis of breast cancer [127], and consists in the apoptosis of tumor cells in the circulation triggered by the loss of interaction with neighboring cells and the extracellular matrix [128,129]. Therefore S-nitrosylation of Src maybe also regulating anoikis resistance in breast cancer.

5. Conclusions

In breast cancer, S-nitrosylation is an important post-translational modification that regulates endothelial and tumoral function modulating different pathways that lead to metastasis. S-nitrosylation in tumor cells activates different pathway leading to EMT, migration and invasion. In endothelial cells, S-nitrosylation modulates endothelial permeability, angiogenesis, adhesion of tumor cells and extravasation. S-nitrosylation of the adherens junction complex seems to be a common target in tumor and endothelial cells. In tumor cells S-nitrosylation of the adherens junction may contribute to EMT and increased migration of tumor cells; in endothelial cells S-nitrosylation of this complex may modulate endothelial permeability contributing to angiogenesis, intra and extravasation of tumor cells (Fig. 4). The discovery and development of specific inhibitors of S-nitrosylation may be a potent therapeutic strategy to treat breast cancer metastasis and other malignancies where S-nitrosylation plays a role. In this regards there is a clinical trial studying the effect of the combined therapy of L-NMMA plus docetaxel in breast cancer patients with refractory locally advanced or metastatic triple negative (L-NMMA Plus Docetaxel in Refractory Locally Advanced or Metastatic Triple Negative Breast Cancer Patients). Other clinical trials are studying the effect of auranofin, an inhibitor of thioredoxin reductase (TRx-R), that decreases the S-nitrosylation levels. The effects of auronofin is been studying in glioblastoma, ovarian, primary peritoneal, fallopian tube cancer, non-small cell lung cancer or small cell lung cancer ("A Proof-of-concept Clinical Trial Assessing the Safety of the Coordinated Undermining of Survival Paths by 9 Repurposed Drugs Combined With Metronomic Temozolomide (CUSP9v3 Treatment Protocol) for Recurrent Glioblastoma"; Auranofin in Treating Patients With Recurrent Epithelial Ovarian, Primary Peritoneal, or Fallopian Tube"; "Auranofin and Sirolimus in Treating Participants With Ovarian Cancer; "Sirolimus and Auranofin in Treating Patients With Advanced or Recurrent Non-Small Cell Lung Cancer or Small Cell Lung Cancer").

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