The role of nitric oxide in tumour progression

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Abstract | Nitric oxide (NO) and nitric oxide synthases are ubiquitous in malignant tumours and are known to exert both pro- and anti-tumour effects. We summarize our current understanding of the role of NO in tumour progression, especially in relation to angiogenesis and vascular functions. We also discuss potential strategies for cancer treatment that modulate NO production and/or its downstream signalling pathways.

S-nitrosylation
A post-translational modification of protein by the covalent addition of a nitrogen monoxide group to the thiol side chain of a cysteine residue of a protein or peptide. It is used in mammalian cells to convey several specific signals that are elicited by nitric oxide.

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Nitric oxide (NO) is a multifunctional gaseous molecule and a highly reactive free radical. It is synthesized from L-arginine, NADPH and oxygen by NO synthase (NOS). As a signalling molecule, NO regulates various physiological and pathophysiological processes, such as vascular functions (angiogenesis, blood flow, vascular permeability, leukocyte–endothelial interaction, platelet aggregation and microlymphatic flow), neurological functions (neurotransmission and development of the nervous system) and, at relatively high concentration, cytotoxic functions (cytostasis and cytolysis). Interestingly, various studies have shown that NO can both promote and inhibit tumour progression and metastasis. The effects of NO in tumours seem to depend on the activity and localization of NOS isoforms, concentration and duration of NO exposure, and cellular sensitivity to NO. In this Review, we summarize our current understanding of various roles of NO in tumour progression, with a focus on vascular functions. We also discuss potential strategies to treat cancer by modulating NO production and/or its downstream signalling.

Mechanisms of NO production and signalling

There are three isoforms of NOS: neuronal NOS (nNOS, also known as NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). nNOS and eNOS are constitutively expressed (predominantly in neuronal cells and vascular endothelial cells, respectively) and are therefore also referred to as constitutive NOS (cNOS) (FIG. 1). cNOS activity is dependent on the concentration of cytosolic calcium (Ca^{2+}), which increases upon various physiological stimuli and facilitates the binding of calmodulin to cNOS[1]. The calmodulin-bound active cNOS dimer produces NO from L-arginine in the presence of co-factors. In addition, eNOS activity is regulated by intracellular localization through trafficking to caveolae, interaction with regulatory molecules such as the stimulator heat-shock protein 90 and the inhibitor caveolin 1, and phosphorylation (at serines 617, 635 and 1179 for activation and at threonine 497 for inhibition)[2]. Conversely, iNOS is transcriptionally regulated and induced by inflammatory cytokines, endotoxin, hypoxia and oxidative stress. iNOS is not dependent on intracellular calcium levels and produces more NO than cNOS[1].

Expression of NOSs and production of NO have been detected in several established human tumours (Supplementary information S1 (table)). Tumour cells often express iNOS and in some cases eNOS and nNOS, depending on tumour type and stage. Tumour vascular endothelial cells predominantly express eNOS. Tumour-associated stromal fibroblasts and immune cells commonly express iNOS. In many tumours, the expression levels and activities of the different NOSs are increased compared with corresponding normal tissues. The effect of NO on tumour progression depends on its source (tumour cells or stromal cells), type and the activity of NOSs.

Many of the physiological processes that are promoted by NO, which include smooth-muscle relaxation, neurotransmission, inhibition of platelet aggregation and adhesion, are mediated by the NO–cGMP signalling pathway[3] (BOX 1). cGMP-dependent protein kinases, cyclic-nucleotide-gated ion channels and cGMP-regulated phosphodiesterases mediate several cellular effects. Post-translational modification of proteins, mainly through *S*-nitrosylation of cysteine thiol residues, is another emerging mechanism of NO signalling that mediates several physiolgocial functions[4] (BOX 1). NO and NO metabolites such as nitrite, nitrate, *S*-nitrosothiols, nitrosamines and peroxynitrite also have an important role in mediating many of the cytotoxic and/or genotoxic effects of NO, such as inhibition of mitochondrial respiration, protein and DNA damage that results in gene mutation, loss of protein function, necrosis and apoptosis[5,6].

At a glance

- Nitric oxide (NO) is synthesized by nitric oxide synthases (NOSs), which are ubiquitously expressed in malignant tumours. NO regulates several physiological processes through the soluble-guanylyl-cyclase–cGMP pathway and *S*-nitrosylation, and has cytotoxic and genotoxic effects at high concentrations.
- Tumour-cell-derived NO promotes tumour progression by induction of tumour-cell invasion, proliferation and the expression of angiogenic factors. The inducible isoform of NOS (iNOS), which produces high concentrations of NO, mediates neoplastic transformation in oncogene- and chemical-induced tumorigenesis models, although conflicting results are reported in the literature. Conversely, the transfection of iNOS-expressing constructs into NO-sensitive tumour cells inhibits tumour growth and metastasis.
- Host stromal-cell-derived NO, which is synthesized by iNOS, inhibits growth of NO-sensitive tumours but promotes growth of NO-resistant tumours.
- NO that is predominantly synthesized by endothelial NOS (eNOS) in vascular endothelial cells promotes angiogenesis directly and functions both upstream and downstream of angiogenic stimuli. Moreover, NO mediates recruitment of perivascular cells and, therefore, remodelling and maturation of blood vessels. NO that is synthesized by eNOS promotes tumour progression through the maintenance of blood flow, induction of vascular hyperpermeability and reduction of leukocyte–endothelial interactions.
- Induction of NO signalling can induce direct tumour-cell cytotoxicity or sensitize tumour cells to other treatments such as radiation. Conversely, blockade of NO signalling can inhibit neoplastic transformation, tumour angiogenesis and blood flow.
- Expression, activity and localization of NOS isoforms, concentration and duration of NO exposure, and cellular sensitivity to NO are important determinants of NO function. Further *in vivo* tumour studies with high spatial and temporal resolution should resolve conflicting issues in NO biology and guide the manipulation of NO signalling for future clinical use.

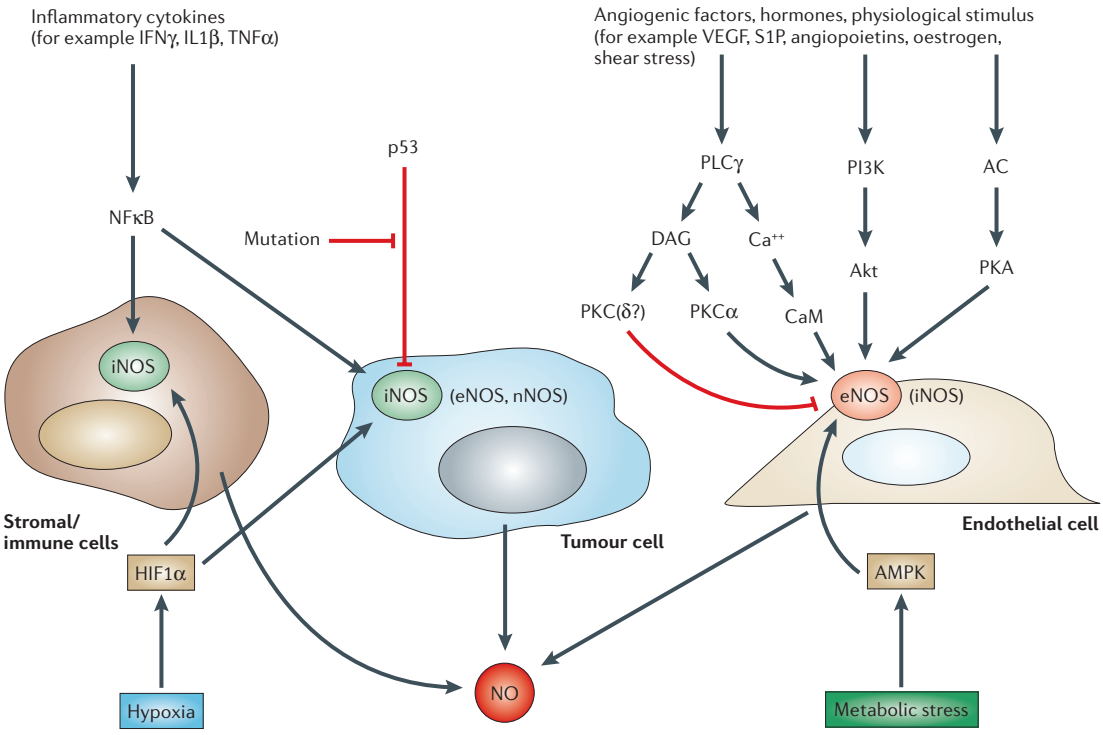
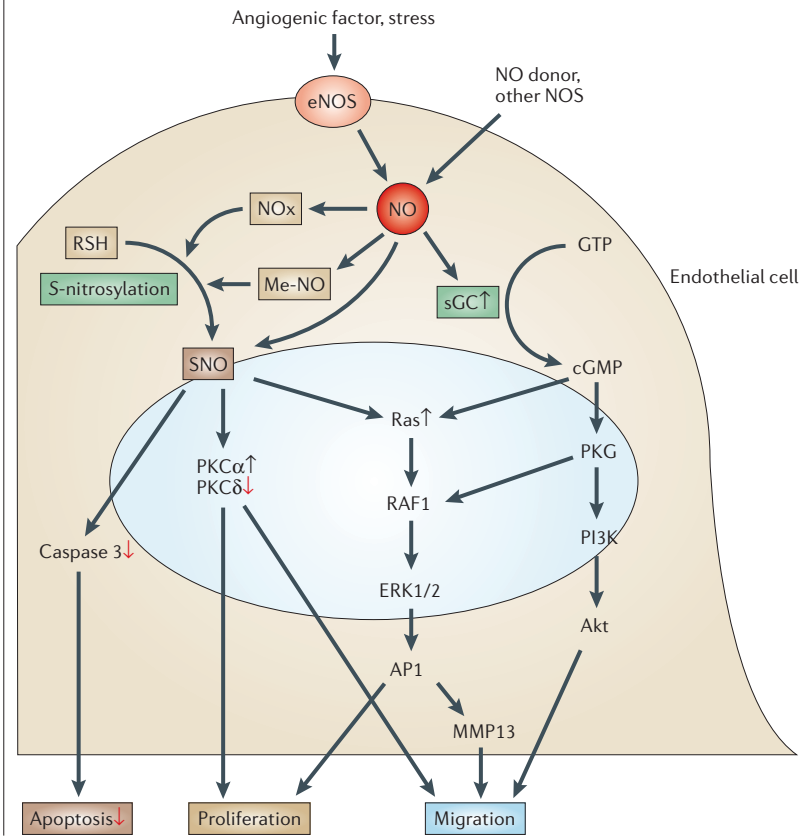


Figure 1 | **Production of nitric oxide (NO) in tumours.** Tumour cells often express inducible NO synthase (iNOS) and in some cases endothelial NOS (eNOS) and neuronal NOS (nNOS). Tumour vascular endothelial cells predominantly express eNOS. Tumour-associated stromal fibroblasts and immune cells express iNOS. Inflammatory cytokines such as interferon-γ (IFNγ), interleukin 1β (IL1β) and tumour necrosis factor-α (TNFα) induce iNOS expression through nuclear factor κB (NFκB). Hypoxia also induces iNOS through hypoxia-inducible factor 1α (HIF1α). Whereas wild-type tumour suppressor p53 inhibits iNOS expression, mutant p53 fails to do so. Angiogenic factors such as vascular endothelial growth factor (VEGF), sphingosine-1-phosphate (S1P), angiopoietins, sex hormones and shear stress activate eNOS in vascular endothelial cells through adenylate cyclase (AC)–protein kinase A (PKA), phosphoinositide 3-kinase (PI3K)–Akt, phospholipase Cγ (PLCγ)– diacylglycerol (DAG)–protein kinase Cα (PKCα) and PLCγ–cytosolic calcium (Ca^{2+})–calmodulin (CaM) pathways. Metabolic stress also activates eNOS through AMP kinase (AMPK). NO is produced by all these different sources in tumours.

Box 1 | **Signalling pathways for endothelial NO-mediated angiogenesis**

Angiogenic stimuli induce nitric oxide (NO) production by endothelial NO synthase (eNOS) in endothelial cells[2]. In endothelial cells, endogenous and/or exogenous NO triggers multiple signalling pathways through *S*-nitrosylation and/or cGMP. *S*-nitrosothiol (SNO) can be formed by NO through three separate pathways: a direct reaction that is followed by electron abstraction; through auto-oxidation of NO (NOx); or through catalysis at metal centres (Me-NO)[151] (see figure). For example, *S*-nitrosylation of Cys163 of caspase 3 (REF. 152) and Cys118 of p21Ras[153] inhibits and stimulates their activity, respectively. Caspase 3 inhibition results in decreased apoptosis and Ras activation results in increased proliferation and migration of endothelial cells[47,53,152]. NO also activates protein kinase Cα (PKCα) and inhibits PKCδ, which results in increased endothelial cell proliferation and migration[53,154]. The soluble guanylyl cyclase (sGC)–cGMP pathway also has an important role in NO-mediated angiogenesis. Binding of NO to the sixth coordinating position of the haem iron of sGC induces a conformational change that results in 200-fold activation of the enzyme and increased synthesis of cGMP[3]. NO-induced cGMP activates Ras and cGMP-dependent protein kinase (PKG). PKG interacts with Raf. The Ras–Raf–MEK (MAPK (mitogen-activated protein kinase) or ERK kinase)–ERK (extracellular signal-regulated protein kinase) pathway then increases the DNA binding of activator protein 1 (AP1), which results in increased cell proliferation and migration[47,53,54]. NO-induced endothelial-cell migration seems to be mediated by PKG through the production of matrix metalloproteinase 13 (MMP13) by ERK[49,155] and by Akt activation through phosphoinositide 3-kinase (PI3K)[48].



Role of NO in neoplastic transformation

Neoplastic transformation is a key initial step in cancer. Chronic inflammation and continuous exposure to moderate-to-high concentrations of NO that is produced by iNOS are thought to promote neoplastic transformation (FIG. 2). NO and NO-derived reactive nitrogen species induce oxidative and nitrosative stress, which results in DNA damage (such as nitrosative deamination of nucleic acid bases, transition and/or transversion of nucleic acids, alkylation and DNA strand breakage) and inhibition of DNA repair enzymes (such as alkyl transferase and DNA ligase) through direct or indirect mechanisms[5,6]. Mutations in the gene that encodes the tumour suppressor p53, or *S*-nitrosylation of caspases, can produce apoptosis-resistant cells and facilitate the further accumulation of mutations and subsequent clonal selection[5,7]. A series of studies has also indicated that NO that is produced by iNOS can initiate and/or promote tumorigenesis[8,9] (TABLE 1). For example, mice with mutations in both adenomatous polyposis coli (*Apc*) and *iNOS* showed fewer adenomatous polyps in the small and large intestines compared with mice with the mutation in *Apc* alone[10]. In addition, *iNOS*$^{-/-}$ mice showed decreased incidence of gastric carcinogenesis that is induced by *Helicobacter pylori*[11]. Moreover, genetic disruption of *iNOS* produced an 80% reduction in urethane-induced lung tumour formation and lower levels of vascular endothelial growth factor (VEGF)[12]. The expression of the Polyoma middle T-antigen (PyV-mT) under the control of the mouse mammary tumour virus long terminal-repeat enhancer/promoter induces mammary tumorigenesis in the transgenic mice. PyV-mT-induced mammary hyperplasia was delayed when the mice were crossed into an *iNOS*-deficient background[13]. Furthermore, tumours in *PyV-mT*/*iNOS*$^{-/-}$ mice were more differentiated than the tumours that were found in *PyV-mT* mice. These findings identify iNOS as a target for tumour chemoprevention. Indeed, an iNOS inhibitor (aminoguanidine) reduced azoxymethane-induced colon cancer[14] and tumorigenesis in *Apc*-mutant mice[10].

By contrast, there are several studies that show inhibitory effects of NO on tumorigenesis[8,9]. DNA damage and/or modification that is produced by NO induces the accumulation of wild-type p53 and activation of poly(ADP-ribose) synthase, and results in death of transformed cells[15,16]. Alternatively, relatively low levels of NO upregulate DNA-dependent protein kinase (DNA–PK), which maintains the integrity of the genome[17]. Tumour necrosis factor-α (TNFα) can induce transformation of JB6 mouse epidermal cells. This process was inhibited by an NO donor (an NO-releasing agent) whereas an NOS inhibitor enhanced it[18]. Contrary to previous studies, Scott *et al.* showed increased intestinal adenomas in *Apc* and *iNOS* double-mutant mice compared with *Apc*-mutant mice[19]. Furthermore, a recent study showed that iNOS-derived NO suppresses lymphomagenesis even in a *p53*$^{-/-}$ background by promoting apoptosis and decreasing tumour cell proliferation[20]. Therefore, it seems likely that NO has context-dependent effects on neoplastic transformation. Both epithelial cells and infiltrated immune cells express iNOS in these tumorigenesis models, although their relative contributions to pro- or anti-tumorigenic effects are difficult to distinguish. The expression level, duration and timing of NO delivery, the microenvironment, the genetic background and the cell type might determine NO sensitivity and the overall effect of NO.

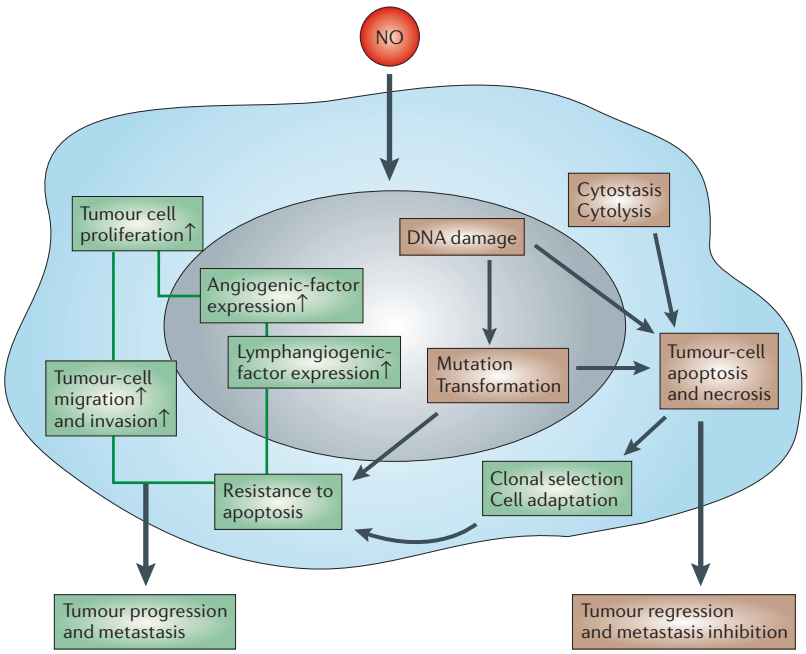


Figure 2 | **Mechanisms of action of NO on tumour cells.** Nitric oxide (NO) can induce both tumour progression and metastasis (green), and tumour regression and inhibition of metastasis (brown) depending on the dose and duration of NO exposure and on cellular sensitivity to NO. NO promotes tumour progression and metastasis by direct induction of tumour-cell proliferation, migration and invasion, and indirectly through the expression of angiogenic and lymphangiogenic factors in tumour cells. On the other hand, the cytotoxic effects of NO that are typically induced by high doses promote DNA damage, gene mutation and tumour-cell death, which result in tumour regression and inhibition of metastasis. However, gene mutation and/or transformation together with cell death of wild-type cells could contribute to clonal selection of adapted cells and the acquisition of apoptosis resistance, and therefore promote tumour progression.

Tumour-cell-derived NO and tumour progression

Several established human tumour cells express iNOS (as well as, in some cases, eNOS and nNOS) and there is clinical evidence that NO that is derived from tumour cells can both promote and inhibit further progression of these tumours depending on the context (Supplementary information S1 (table)). In experimental models, a genetic induction of iNOS in tumour cells resulted in an increase in tumour growth whereas transduction of antisense *iNOS* into tumour cells reduced tumour growth[15,21,22] (TABLE 1). Several mechanisms have been proposed to explain how NOS expression in tumour cells promotes tumour progression (FIG. 2): first, iNOS and eNOS were shown to enhance migration and invasion of breast cancer and colon cancer cells through soluble guanylate cyclase (sGC) and the mitogen-activated protein kinase (MAPK) pathway[23–25]; second, iNOS was found to be associated with increased cell proliferation in T-lymphoma cells[26]; third, NO from murine melanoma cells reduced the lymphocyte reaction in tumour-bearing mice, which indicates an escape from immunosurveillance through NO-induced immune-cell dysfunction[27]; and last, several studies have shown that iNOS induction in tumour cells promotes angiogenesis (by upregulating VEGF expression), which increases microvascular density and tumour progression[15,21]. Histological examinations reveal a relationship between high angiogenic activity or high VEGF expression and iNOS expression in human brain, head and neck, lung, breast, stomach and colon tumours (Supplementary information S1 (table)). These findings indicate that tumour-cell-derived NO mediates tumour angiogenesis, invasion and growth.

However, there have been several studies that offer conflicting observations. The histological examination of several human cancers has revealed that iNOS expression does not show any correlation with tumour progression. In some cases, iNOS expression in tumour cells was inversely correlated with tumour stage, grade and progression, and positively correlated with apoptosis and patient survival (Supplementary information S1 (table)). Induction of iNOS in tumour cells with wild-type p53 resulted in reduced tumour growth[15]. In addition, although many observations indicate that iNOS is important for angiogenesis, others have shown that iNOS expression does not correlate with VEGF expression, microvascular density or tumour progression in some human tumour tissues (Supplementary information S1 (table)). It is still unclear how NO might inhibit tumour angiogenesis, but Nunokawa *et al.* have demonstrated that high NO production that results from iNOS induction can inhibit the proliferation of endothelial cells and vascular smooth-muscle cells[28]. Furthermore, the high concentration of NO that is produced by iNOS induces apoptosis. It has been shown that transduction of iNOS in tumour cells induces apoptosis and inhibits tumour growth and metastasis in several mouse tumour models[15,29–32] (TABLE 1). Tumour-cell-derived NO prevents platelet aggregation[33]. A metastatic human colorectal carcinoma cell line has lower iNOS activity but higher platelet aggregation compared with a non-metastatic tumour line that is derived from the same patient. Platelets are known to enhance metastasis[34]. They store angiogenic factors, stimulate angiogenic vessel growth[35] and also mediate retention of metastasizing tumour cells in blood vessels after the initial entrapment[36]. Taken together, these data indicate that iNOS-derived NO in tumour cells also has an anti-tumorigenic role.

These discrepancies might originate from differences in tumour type, host tissue or tumour models. Local NO levels and cellular responsiveness to NO might be an important determinant in the overall tumour response to NO. However, in most of these studies, spatial and temporal distribution, and local concentration of NO in tumours were not determined. Interestingly, Ambs *et al.* showed that the response to iNOS induction was dependent on the status of p53 in tumour cells. iNOS induction increased VEGF expression, angiogenesis and tumour growth in p53-mutant tumour cells but decreased the growth of tumours with wild-type p53 (REF. 15). Moreover, Wang *et al.* used several murine pancreatic adenocarcinoma cell lines that differentially expressed iNOS to show that moderate, but not high, expression of iNOS in tumour cells correlated with high metastatic potential and rapid tumour growth[37]. Le *et al.* recently showed an NO-dose-dependent increase in apoptosis and a decrease in tumour growth of human

Electron abstraction
A chemical reaction or transformation that results in the bimolecular removal of an electron from a molecular entity. Molecular scavenging of electrons can produce free radicals. Free radicals are reactive species that can modify DNA, lipids and proteins.

Caspases
A family of cysteine proteases. Activated caspases initiate the apoptotic signal or execute a phase of apoptosis when cells receive an apoptosis-inducing signal. Apoptotic cell death is inhibited by *S*-nitrosylation in the catalytic site of caspases.

Table 1 | ***In vivo* causal relationship between nitric oxide (NO) and tumour progression**

Role of NO	Methods of NO modulation	Findings	References
Induces tumorigenesis	*iNOS*$^{-/-}$ mice	Decreased tumorigenesis in *Apc*-mutation-induced colonic polyposis, Polyoma virus middle-T-antigen-induced breast hyperplasia, *Helicobactor*-induced gastric carcinogenesis and urethane-induced lung tumorigenesis	10–13
Inhibits tumorigenesis	*iNOS*$^{-/-}$ mice	Increased tumorigenesis in *Apc*-mutation-induced colonic polyposis, lymphomagenesis and sarcomagenesis	19,20
Promotes angiogenesis in tumours	NOS inhibitors (L-NAME, L-NMMA, cavtratin)	Decreased angiogenesis and tumour growth in murine melanomas, murine lung and breast tumours, and human head and neck tumour and hepatoma xenografts	61,62,132,133
Tumour-derived NO induces angiogenesis	*iNOS* gene transduction in human colon cancer cells	Enhanced VEGF expression in tumour cells, xenograft neovascularization and growth	15,21
Host-derived NO induces angiogenesis	*eNOS*$^{-/-}$ mice	Reduced vessel number, length, and branching in murine melanomas	62
	iNOS$^{-/-}$ mice	Reduced VEGF expression, tumorigenesis and tumour growth in murine melanomas and lung tumours	12,43
Host-derived NO recruits perivascular cells	*eNOS*$^{-/-}$ mice; NOS inhibitor	Impairment of pericyte recruitment in murine melanomas	62
Maintains tumour blood flow	NOS inhibitors (L-NMMA, L-NAME, L-NNA)	Decreased tumour blood flow in murine gliomas, melanomas, sarcomas, breast and colon cancers, and human colon cancer xenografts	89–92
Induces hyperpermeability	NOS inhibitors (L-NAME, cavtratin)	Reduced vascular permeability in murine sarcomas, breast and lung cancers	61,89,92,101
Decreases leukocyte–endothelial interaction	NOS inhibitor (L-NAME)	Increased leukocyte–endothelial interaction in murine breast cancer	89
Tumour-derived NO promotes tumour growth	Antisense reduction of *iNOS* expression	Decreased tumour growth in murine gliomas *in vivo* (but did not affect *in vitro* cell proliferation)	22
	iNOS gene transduction in p53-mutated human colon cancer cells	Enhanced VEGF expression in tumour cells; xenograft neovascularization and growth	15,21
Host-derived NO promotes tumour growth	*iNOS*$^{-/-}$ mice	Reduced VEGF expression, tumorigenesis and tumour growth in murine melanomas and lung tumours	12,43
Tumour-derived NO inhibits tumour growth	*iNOS* gene transduction	Increased p21 and apoptosis, and decreased tumour growth in murine melanoma and sarcoma, and human renal, ovary, prostate and breast cancer xenografts	15,31,32
Host-derived NO inhibits tumour growth	*iNOS*$^{-/-}$ mice	Murine fibrosarcomas grew faster	42
Promotes metastasis	NOS inhibitors (L-NAME, L-NNA)	Inhibits experimental lung metastasis of iNOS-expressing murine breast cancer and melanoma cells	27,123
Host-derived NO promotes metastasis	*iNOS*$^{-/-}$ mice with experimental metastasis of murine breast cancer and melanoma cells	Decreased size and incidence of metastases, and longer survival	44,45
Tumour-derived NO inhibits metastasis	*iNOS* gene transduciton	Abrogated experimental metastasis of murine melanoma and fibrosarcoma, and human colon and renal carcinoma cells	29–32
Vasculature-derived NO inhibits metastasis	NOS inhibitors (L-NAME, L-NMMA)	Upon entrapment of metastasizing murine melanoma and hepatoma cells in the liver and lung microvessels, NO was released and induced tumour-cell toxicity, which was blocked by the NOS inhibitors	110–112
	eNOS$^{-/-}$ mice	Decreased NO production and apoptosis of metastasizing murine melanoma cells in the lung vasculature	112
Host-derived NO inhibits metastasis	*iNOS*$^{-/-}$ mice with experimental metastasis of murine breast cancer and melanoma cells	Increased metastasis incidence and size; (tumour cells are sensitive to NO-dependent cytotoxicity)	42,44

Apc, adenomatous polyposis coli; eNOS, endothelial NOS; iNOS, inducible NOS; L-NAME, Nω-nitro-L-arginine-methyl ester; L-NMMA, N^G-monomethyl-L-arginine; L-NNA, Nω-nitro-L-arginine; nNOS, neuronal NOS; NOS, NO synthase ; VEGF, vascular endothelial growth factor.

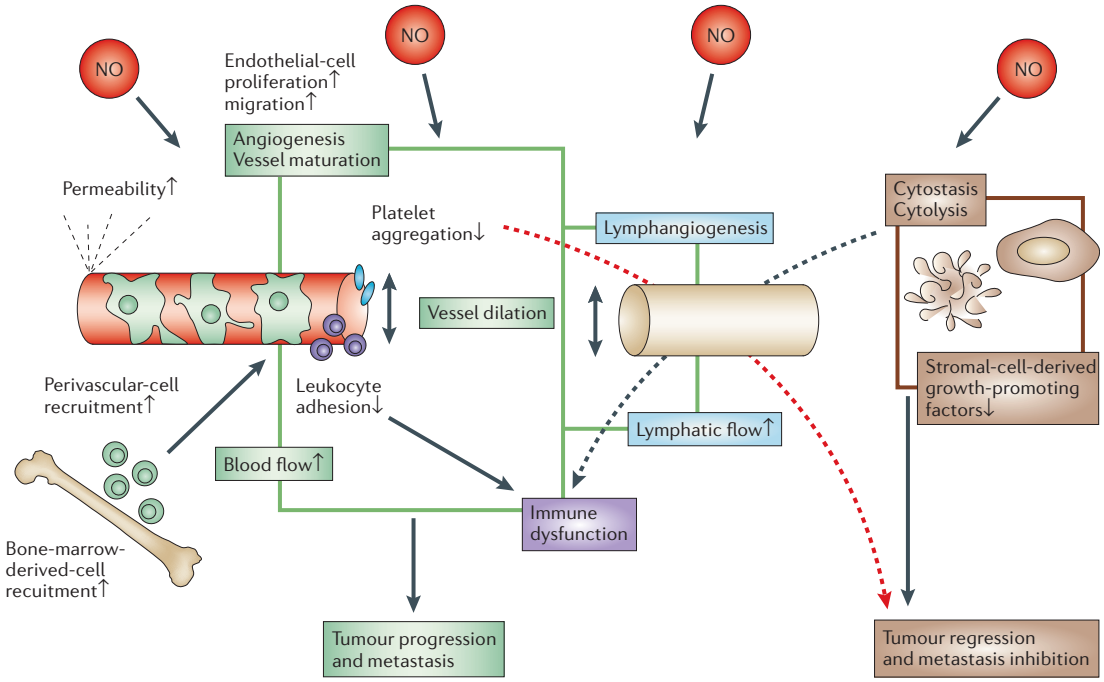


Figure 3 | **Mechanisms of action of NO on host cells.** Nitric oxide (NO) promotes tumour progression and metastasis (green boxes) by maintaining tumour blood flow through angiogenesis, vessel maturation and vessel dilation. NO induces vascular endothelial-cell proliferation and migration, bone-marrow-derived-cell recruitment and perivascular-cell recruitment. Induction of vascular hyperpermeability by NO contributes to formation of tumour extracellular matrix and augments angiogenesis and tumour growth. Decreased leukocyte adhesion and the cytotoxic effects of NO might allow tumours to evade immune-cell attack. Lymphangiogenesis and maintenance of lymph flow (blue boxes) might also contribute to lymphatic metastasis. On the other hand, the cytotoxic effects of NO (brown boxes) reduce the levels of various growth-promoting factors that are derived from non-neoplastic stromal cells and might therefore result in tumour regression. As platelet aggregation contributes to metastasis, its reduction by NO might also inhibit metastasis.

tumour xenografts by transducing tumour cells with serially mutated *iNOS* constructs that produce mutant iNOS proteins with different degrees of enzyme activity[31]. Abundant expression of iNOS can lead to toxic levels of NO and tumour-cell death, whereas modest NO production might be insufficient to produce cytotoxic effects. Continuous exposure to low levels of NO produces NO resistance, which might allow tumour progression even in the presence of typically cytotoxic levels of NO[38]. Although exogenous *iNOS* transfer (which produces NO at high levels) induces anti-tumour activity, endogenous iNOS expression in tumour cells seems to promote tumour growth, angiogenesis, invasion and metastasis. Therefore, specific inhibition of iNOS might be a strategy for cancer treatment. However, these apparent contradictory findings in animal models have indicated that NO might have dual effects in cancer and highlight the importance of careful scrutiny of the use of iNOS inhibitors as anti-tumour reagents in humans.

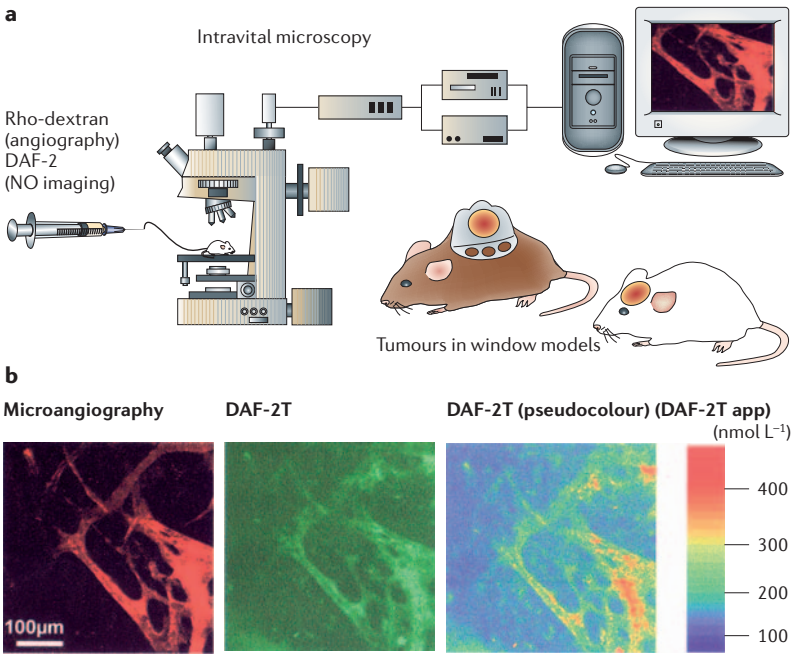
Stromal-cell-derived NO and tumour progression

Role of stromal iNOS in tumour progression. Tumour tissue consists not only of tumour cells but also of non-neoplastic stromal cells such as vascular cells, fibroblasts and immune cells. In many human tumours, upregulation of iNOS has been shown in stromal fibroblasts and immune cells in addition to or independent of its expression in tumour cells (Supplementary information S1 (table)). Isolated tumour-associated macrophages or cytokine-activated fibroblasts have tumoricidal activity through iNOS-derived NO[39–41]. By growing *iNOS*-deficient tumour cells in *iNOS*$^{-/-}$ and wild-type mice, Wei *et al.* showed that host iNOS suppressed growth and metastasis of methylcholanthrene-induced murine fibrosarcomas[42] (TABLE 1). These results indicate that NO that is produced by stromal cells has tumoricidal activity and host iNOS might suppress tumour growth and metastasis (FIG. 3).

By contrast, several similar studies produced conflicting results. For example, Knopka *et al.* showed lower VEGF expression and slower growth of B16 melanoma in *iNOS*$^{-/-}$ mice, which indicates that iNOS in tumour stroma promotes tumour growth through induction of VEGF expression and angiogenesis[43]. The discrepancy in the role of iNOS in tumour stroma might be due to different tumour microenvironments and NO sensitivity among various types of tumour cells. Shi *et al.* demonstrated that host *iNOS* deletion increased metastatic tumour growth of M5076 ovarian sarcomas in the lung, whereas decreased metastasis of B16–BL6 melanomas was observed in *iNOS*$^{-/-}$ mice[44]. M5076 cells are sensitive and B16–BL6 cells are insensitive to macrophage-induced NO-mediated cytotoxicity. In the latter case, host iNOS promoted tumour progression for the NO-insensitive

Box 2 | **Visualization of NO in tumours (intravital DAF-2T imaging)**

a | Tumour tissue distribution of nitric oxide (NO) can be visualized *in vivo* using an NO-sensitive fluorescent probe, DAF-2 (REFS 62,112,156). In the presence of oxygen, NO converts DAF-2 to DAF-2T. DAF-2T is 180 times more fluorescent than DAF-2 (REF. 157). Intravital microscopy of *in vivo* tumours can be carried out by growing them in transparent window models or with careful surgical preparation[158]. After intravenous injection of DAF-2, DAF-2T fluorescence in tumours increases in a time-dependent manner. **b** | Example of NO imaging in a B16–F10 murine melanoma that is grown in a mouse cranial window. The lefthand panel shows microangiography by intravenous injection of tetramethylrhodamine-dextran (with a molecular weight of 2 million). The middle panel shows a representative DAF-2T microfluorograph that was captured 60 minutes after the loading of DAF-2. The righthand panel shows a pseudocolour representation of the DAF-2T microfluorograph. The colour bar in the bottom right shows a calibration of the fluorescence intensity with known concentrations of DAF-2T (DAF-2 Tapp). The scale bar represents 100μm in all three panels.



tumour cells. Cellular origin and tissue localization of NO production might also determine the effect of NO on tumour progression (BOX 2). Gauthier *et al.* showed that iNOS expression in tumour cells reduces, whereas iNOS expression in host stromal cells increases, the development of metastatic nodules of murine breast carcinomas[45].

Role of NO in tumour angiogenesis. Angiogenesis is essential for tumour growth and metastasis[46]. NO has been shown to mediate angiogenesis by direct and indirect mechanisms (FIG. 3). First, NO exposure increases DNA synthesis, cell proliferation and migration of endothelial cells through the sGC–cGMP pathway as well as through *S*-nitrosylation[47–49] (BOX 1). Anti-angiogenic effects of NO have also been reported[50–52]. Recent studies indicate this discrepancy might be explained by differences in concentration and duration of NO exposure. Low concentrations of NO increase, but high concentrations inhibit, phosphorylation of protein kinase C (PKC), extracellular-signal-regulated protein kinase (ERK) and JUN, and the binding activity of activator protein 1 [53,54].

Second, NO has been shown to mediate the function of many angiogenic factors[55,56]. VEGF, sphingosine-1-phosphate, angiopoietins, oestrogen, shear stress and metabolic stress activate eNOS through phospholipase-C–Ca^{2+}–calmodulin binding and phosphoinositide 3-kinase (PI3K)–AKT-induced and adenylate-cyclase–protein-kinase-A-induced phosphorylation[2] (FIG. 1). eNOS also mediates VEGF- and angiopoietin-1-induced angiogenesis *in vivo*[57,58]. Furthermore, anti-angiogenic factors exert their effects through inhibition of eNOS–NO signalling. Endostatin dephosphorylates eNOS at serine 1177 through protein phosphatase 2A and an NO donor reverses the inhibitory effect of endostatin on endothelial-cell migration[59]. Thrombospondin 1 (TSP1) inhibits cGMP formation and the resultant downstream signalling that is induced by NO[60]. Along with these findings, increased eNOS expression has been noted in the vasculature of various human tumour tissues when compared with normal tissue samples, and it has been linked with increased tumour angiogenesis, high vascular permeability and metastasis (Supplementary information S1 (table)). Selective inhibition of eNOS, genetically or with a pharmacological agent, has been shown to inhibit tumour angiogenesis[61,62].

Third, NO is an important modulator of the expression of endogenous angiogenic factors. An NO donor induces VEGF expression[63] and coronary-vein endothelial-cell proliferation through the expression of basic fibroblast growth factor[64]. NO activates the transcription factor hypoxia-inducible factor 1α (HIF1α), which in turn upregulates VEGF, thereby promoting angiogenesis[65,66]. NO induces HIF1α synthesis through MAPK and PI3K under normoxic conditions[67]. It also impairs normoxic degradation of HIF1α by inhibiting the action of prolyl hydroxylases[68]. By contrast, NO has also been shown to inhibit hypoxia-induced HIF1α activation and VEGF expression[69–71]. This discrepancy might be explained by the difference in the duration of exposure to NO. NO-induced HIF1α activation is transient (with a peak at 4 hours) and more than 20 hours of NO treatment decreases HIF1α activity[72,73]. Haem oxygenase 1 also mediates NO-induced VEGF upregulation in human endothelial cells[74]. A recent study found expression of iNOS and/or eNOS in all cases of HIF1α-positive oral squamous-cell carcinomas, which suggests that there is NO-induced HIF1α accumulation and subsequent tumour-promoting effects in cancer[75].

Furthermore, NO inhibits expression of endogenous anti-angiogenic factors. An NO donor reduces TSP1 expression[54] and an NO inhibitor increases angiostatin expression[76] in vascular endothelial cells. Downregulation of TSP-1 by NO depends on cGMP and ERK[54]. NO seems to regulate pro- and anti-angiogenic factors through multiple mechanisms.

Recruitment of bone-marrow-derived cells could be another mechanism by which NO regulates new vessel formation. A growing body of evidence indicates that bone-marrow-derived cells might be recruited into ischaemic and/or neoplastic tissues and participate in new vessel formation. However, the identity, frequency and fate of these cells remain controversial[77–80]. Aicher

et al. showed that defects in angiogenesis and reperfusion in ischaemic tissues of *eNOS*$^{-/-}$ mice could be rescued by injection of stem/progenitor cells that were derived from wild-type mice[81]. However, this failed to correct angiogenic defects if ischaemic insults were applied after the bone-marrow reconstitution, which indicates a defect in the mobilization of stem/progenitor cells from the bone marrow in *eNOS*$^{-/-}$ mice. Angiogenic factors such as VEGF activate matrix metalloproteinase 9 (MMP9) in bone-marrow stromal cells. MMP9 cleaves membrane-bound KIT ligand, and soluble KIT ligand mobilizes stem and progenitor cells from bone marrow[77]. NO increases MMP9 expression through the cGMP pathway[82] and also activates pro-MMP9 through *S*-nitrosylation[83]. VEGF-induced mobilization of endothelial progenitor cells, 5-fluorouracil-induced MMP9 activation and soluble KIT ligand production were all shown to be defective in *eNOS*$^{-/-}$ mice[81]. However, baseline and ischaemia-induced mobilization of endothelial progenitor cells was unaffected[81,84]. The contribution of NO to tumour angiogenesis through bone-marrow-derived-cell recruitment has yet to be shown. Further studies are needed to clearly elucidate the role of NO in tumour angiogenesis through this mechanism.

Role of NO in tumour vessel maturation and functions. Mature blood vessels consist of two distinct types of cell, endothelial cells and perivascular cells. Recruitment of perivascular cells (such as pericytes and vascular smooth-muscle cells) is an important step in angiogenesis, vascular morphogenesis and vessel maturation[85]. Long-lasting, stable blood vessels can be formed *in vivo* by fostering interactions between vascular endothelial cells and perivascular cells[86]. Recent studies have revealed the role of NO in vessel maturation through perivascular-cell recruitment[62,84]. In B16 murine melanomas, reduced perivascular-cell coverage was found in variants that expressed low amounts of NOS, through NO blockade and in *eNOS*$^{-/-}$ (but not *iNOS*$^{-/-}$) mice[62]. Reduced perivascular-cell coverage was also observed in ischaemic limbs of *eNOS*$^{-/-}$ mice[84]. These findings indicate that NO that is produced by eNOS in vascular endothelial cells promotes perivascular-cell recruitment and, as a result, vessel remodelling and maturation (FIG. 3).

In addition to the function of NO in the modification of vessels in response to chronic stimuli, NO has been shown to regulate vascular function in response to acute stimuli (FIG. 3). NO was originally discovered as an endothelium-derived relaxing factor because NO that is produced by eNOS relaxes the adjacent vascular smooth-muscle cells through activation of the sGC–cGMP signalling pathway[87,88]. Endogenous NO maintains blood flow by dilatation of arterial vessels. NO similarly regulates tumour blood flow and, consequently, tumour blood flow can be dynamically altered by modulation of NO levels[55,89–92]. However, the tumour vascular response to NO inhibitors or donors is diverse[89] owing to heterogeneous spatial and temporal distribution of NOS and NO. Expression of sGC and phophodiesterases, which degrade cGMP, in tumour perivascular cells could be variable and, therefore, cGMP levels in response to a given concentration of NO in these cells might not be comparable to those that are seen in normal vasculature. These heterogeneities in the tumour vasculature could explain its abnormal function and response to NO.

Vascular permeability is another important component of vessel function. Both positive and negative regulation of vascular permeability by NO have been reported[55,93]. Precise mechanisms that explain this discrepancy are unknown. Increased microvascular protein leakage by NOS-inhibitor treatment was reported in intact normal tissues such as stomach, intestine, liver, spleen, pancreas, kidney and lung. On the other hand, NOS inhibition attenuates vascular permeability induction by cytokines and growth factors such as VEGF, interleukin 2, histamine, leukotriene C_4, ADP, bradykinin, substance P, endotoxin, serotonin and platelet-activating factor[57,94–98]. Low concentrations of constitutive NO might maintain normal blood-vessel integrity whereas increased production of NO under pathological conditions might mediate vascular hyperpermeability. Tumour vessels are known to be leaky owing to structural abnormalities of the vascular wall and the presence in tumours of potent inducers of vascular permeability such as VEGF. High vascular permeability, together with abnormal lymphatic functions, results in elevated interstitial fluid pressure in tumours and poor delivery of therapeutic agents. Furthermore, extravasated plasma proteins such as fibronectin form an optimal provisional matrix for angiogenesis. Induction of vascular permeability by VEGF is mediated by eNOS and its downstream signalling through cGMP, PKC, ERK1 and ERK2 (REFS 57,99,100). It seems that NO-mediated vascular permeability and angiogenesis share the same signalling pathways (BOX 1). Inhibition of NOS has been shown to decrease vascular permeability in various tumour models[61,89,92,101]. More recently, a cell-permeable peptide that contains the eNOS-binding domain of caveolin 1 has been shown to suppress vascular leakage and delay tumour progression in a hepatoma xenograft model by locally blocking eNOS in the tumour vasculature[61,102].

NO has been shown to mediate cell–cell interactions in the vasculature. NOS inhibitors upregulate and NO donors downregulate leukocyte–endothelial interactions through the expression of platelet selectin (P-selectin), intercellular adhesion molecule 1 (ICAM1) and vascular cell-adhesion molecule 1 (VCAM1) in normal vessels[103–105]. Increased p-selectin-mediated leukocyte–endothelial interactions were observed in the mesenteric vessels of *eNOS*$^{-/-}$ and *nNOS*$^{-/-}$ mice but not in *iNOS*$^{-/-}$ mice[106]. Leukocyte adhesion in tumour vessels is usually low and this might limit host immune response and the efficacy of immune therapy in tumours[107]. Reduction in the NO-mediated expression of adhesion molecules might explain low leukocyte–endothelial interactions in the tumour vasculature. Inhibition of NOS increases both rolling and stable adhesion of leukocytes along the tumour vasculature[89]. However, NO inhibits the aggregation of platelets through a cGMP-dependent mechanism[108]. Platelets form aggregates with tumour cells and facilitate their adhesion to vascular endothelial cells and haematogeneous dissemination[109]. The ability

Reperfusion
The restoration of blood supply to an organ or tissue that has been starved of oxygen because of a decrease in normal blood supply. Post-ischaemia reperfusion often leads to the generation of oxygen radicals.

Pericytes
Specialized mesenchymal-like cells that are found in close association with the walls of small blood vessels. Normal pericytes share a basement membrane with vascular endothelial cells and are important for blood-vessel maturation, stabilization, remodelling and function. In tumours, the morphology and function of pericytes are often abnormal.

Extravasation
The exit of molecules or cells from blood vessels to the perivascular region.Here, this term is used for plasma leakage from vessels.

Table 2 | **Potential approaches to tumour treatment by modifying nitric oxide (NO) signalling**

Strategy	Target	Potential approaches	*In vivo* studies that have been reported to date	References
Increasing NO signalling				
Direct cytotoxic agent	Tumour cells	*iNOS* gene transduction	Injection of a plasmid that encodes CMV-promoter-driven *iNOS* into tumour tissues inhibited growth of murine thyroid cancers	113
		Injection of NO-releasing agent, device or cell	Peritumoural injection of microencapsulated iNOS-expressing cells increased FAS/FASL protein levels in tumours and inhibited growth of human colon and ovarian cancer xenografts	114
		Induction of iNOS by cytokines or drugs	Liposomal delivery of lipopeptide CGP31362 and IFNγ induced iNOS expression, NO-dependent tumour-cell apoptosis and regression of murine sarcoma hepatic metastasis	116
Sensitizing agent	Tumour cells	*iNOS* gene transduction	Intratumoural injection of an adenovirus vector that encodes iNOS increased vascular density and radiosensitivity in human colorectal cancer xenografts	126,127
Indirect sensitization through increased blood flow	Blood vessels	Activation of eNOS	Electrical stimulation and low-dose radiation activated eNOS, and increased tumour blood flow, oxygenation and radiation sensitivity in murine liver and lung cancers and fibrosarcomas	129,130
Decreasing NO signalling				
Anti-angiogenesis or tumour blood-flow shut down	Blood vessels	NOS inhibitor	An iNOS-selective inhibitor (1400W) inhibited growth of iNOS-expressing tumours. A non-selective NOS inhibitor (L-NAME) and an eNOS inhibitor (cavtratin) reduced tumour angiogenesis, blood flow, microvascular permeability and growth	61,131, 132,134
		Caveolin 1 gene transduction	Intravenous injection of a plasmid that encodes caveolin 1 and is complexed with cationic lipids reduced tumour blood flow, angiogenesis and growth of murine hepatocarcinomas	135
Anti-lymphangiogenesis or tumour lymph-flow shut down	Lymph vessels	NOS inhibitor	No *in vivo* study is available to date	
Tumour prevention	Tumour cells	NOS inhibitor	iNOS-selective inhibitors (aminoguanidine and 1400W) suppressed tumorigenesis in three models: *Apc*-mutation-induced colonic polyposis, azoxymethane-induced formation of colonic aberrant foci and low-dose irradiation combined with diethylstilbestrol-induced mammary tumorigenesis	10,14, 138,139

Apc, adenomatous polyposis coli; CMV, cytomegalovirus; eNOS, endothelial NOS; FASL, FAS ligand; IFNγ, interferon-γ; iNOS, inducible NOS; L-NAME, Nω-nitro-L-arginine-methyl ester; NOS, NO synthase.

of a tumour cell to aggregate platelets, which correlates with metastatic potential, is inversely proportional to NO production[33]. NO might reduce tumour metastasis by reducing platelet aggregation.

Finally, evidence that indicates that endothelial-cell-derived NO mediates the elimination of disseminated tumour cells is increasing. Intravascular arrest of tumour cells in the liver has been shown to induce a rapid local release of NO that causes apoptosis of the tumour cells[110,111]. Recently, Qiu *et al.* showed that endothelial-cell-produced NO had a cytotoxic effect on disseminating tumour cells[112]. Histological analyses of human cancer specimens also show that eNOS can be overexpressed in the tumour vasculature (Supplementary information S1 (table)). Therefore, host eNOS might present a tumoricidal barrier and protect against tumour-cell dissemination.

Tumour treatment by modulation of NO levels

Modulation of NO signalling might be used for the treatment of tumours. The literature supports both increasing and decreasing NO signalling as a potential strategy. Type and stage of tumour, and the expression, activity, and spatial and temporal distribution of NOS isoforms should be taken into consideration in deciding what kind of approach to use.

Treatment strategies to increase NO signalling. There have been attempts to use NO as a tumoricidal agent in several preclinical studies (TABLE 2). Three approaches have been tested: delivery of the *iNOS* gene, delivery of iNOS-expressing cells and induction of iNOS by an inflammatory cytokine or other agent. Solar *et al.* showed a 35% volume reduction of established murine thyroid cancer in 4 days by intratumoural injection of an iNOS-expressing plasmid vector[113]. Xu *et al.* demonstrated that delivery of iNOS-overexpressing cells to the peritumoural region resulted in increased FAS and FAS-ligand expression, and inhibition of tumour growth in human ovarian cancer and colon cancer xenografts[114]. A problem with these approaches is that iNOS-transduced cells might undergo apoptosis quickly and produce NO for only a limited time. Another problem could be an inadequate supply of NOS substrates. Some tumour cells seem to overexpress arginase[115], which breaks down L-arginine and therefore leads to the depletion of tumour-associated L-arginine. Moreover, inadequate

tumour perfusion because of abnormal tumour vessel structure might lead to a shortage of NOS substrates. Consequently, relatively low levels of NO might induce NO resistance and promote tumour growth rather than cell killing[38].

Alternatively, cytokines and/or chemicals that induce iNOS expression could be used to induce NO-mediated tumour-cell killing. Systemic administration of liposomes that contain immunomodulator lipopeptide CGP31362 and interferon-γ (IFNγ) induced iNOS expression, NO-dependent tumour-cell apoptosis and tumour regression in murine sarcoma hepatic metastases[116]. Lipid A and interleukin 10 also induced iNOS and showed anti-tumour activity in murine colon and breast cancers[117,118]. Additionally, NO has been shown to mediate the anti-tumour-cell activity of various agents such as TNFα, *N*-(4-hydroxyphenyl)retinamide (a synthetic derivative of all-*trans* retinoic acid), short interfering RNA against *STAT3* (signal transducer and activator of transcription 3) and farnesyltransferase inhibitors (α-hydroxyfarnesyphosphonic acid, manumycin A and SCH663360)[119–122]. However, it should be noted that induction of iNOS by lipopolysaccharide (LPS) and IFNγ inhibits tumour cell proliferation *in vitro* but stimulates experimental metastasis of murine breast cancer cells[123]. Systemic injection of LPS also induced upregulation of iNOS, and increased angiogenesis and metastatic tumour growth in a different murine breast cancer model[124]. Different cells and tissues respond to cytokines and/or NO differently and systemic effects of this approach require careful evaluation.

In addition to its direct effect, NO is known to sensitize hypoxic cells to radiation[125]. The mechanism of NO-induced radiosensitization is thought to be through the prevention of subsequent repair of radiation-induced damage to DNA. Intratumoural injection of an iNOS-encoding adenovirus potentiated radiation treatment of human colon cancer xenografts[126,127]. Moreover, iNOS sensitization was more effective in p53-wild-type tumours than p53-null tumours[126].

An increase in tumour vessel density was also observed after treatment with an iNOS-expressing adenovirus[127]. So, increased tumour oxygenation might also participate in NO-induced radiosensitization. In this regard, activation of eNOS in vascular endothelial cells might be an alternative and more effective approach. NO mediates recruitment of perivascular cells to tumour vasculature[62], which is an important step in 'normalizing' chaotic tumour vasculature for the efficient delivery of therapeutic agents and oxygen to tumours[128]. Therefore, activation of vascular eNOS might be a novel strategy to control tumour vessel structure and to potentiate anticancer treatments such as cytotoxic and radiation therapies against solid tumours. Electrical stimulation and low-dose radiation have been shown to activate eNOS, increase tumour blood flow and tumour oxygenation, and enhance radiosensitivity of murine liver and lung cancers and fibrosarcomas[129,130].

Treatment strategies to decrease NO signalling. Although induction of NO signalling in tumours might improve the blood supply and therefore improve the delivery of therapeutic agents, increasing the blood flow is not a desirable outcome early in tumorigenesis. Accumulating clinical and preclinical data demonstrate that NO mediates angiogenesis through direct and indirect mechanisms, and maintains tumour blood flow and the supply of nutrients and oxygen, thereby promoting tumour progression (TABLE 1; Supplementary information S1 (table)). Therefore, inhibition of NO signalling is a potential anti-angiogenic therapeutic strategy. Continuous inhibition of iNOS by a selective inhibitor, 1400W, inhibits the growth of human colon cancers as well as of murine breast cancers that express endogenous iNOS[131]. However, 1400W failed to inhibit murine colon cancers that did not express iNOS at appreciable levels. Constitutively expressed eNOS in vascular endothelial cells might be a more attractive target for anti-angiogenic therapy. Non-selective NOS inhibitors reduce tumour angiogenesis, blood flow and growth in several murine tumours and human tumour xenografts[62,132–134]. More recently, Gratton *et al.* showed that a cell-permeable peptide that is derived from caveolin 1, called cavtratin, reduced vessel density, microvascular permeability and tumour progression in human hepatoma xenografts and murine lung cancers by inhibiting eNOS[61]. Brouet *et al.* showed that intravenous injection of a cationic-lipid-coated plasmid that encodes caveolin 1 led to the selective expression of recombinant caveolin 1 in tumour vasculature, reduced tumour blood flow and decreased tumour growth. This was presumably through inhibition of pro-angiogenic and vasodilatory effects of NO that was derived from eNOS in vascular endothelial cells[135]. Although cavtratin does not inhibit iNOS, ectopic expression of caveolin 1 decreased iNOS activity by direct association with iNOS, thereby causing its degradation through the proteasome pathway in human colon cancer cells[136]. In addition to eNOS, caveolin 1 also associates with receptor tyrosine kinases and G-protein-coupled receptors in endothelial cells and other cell types such as vascular smooth-muscle cells[137]. The biological consequences of the induction of caveolin 1 or the delivery of cavtratin seem to be more complex than simple eNOS downregulation. Further studies are required prior to the clinical application of this strategy.

Pre-clinical studies indicate that NO downregulation might be of value in chemoprevention. Prevention of tumorigenesis by selective inhibitors of iNOS has been reported in three different tumorigenesis models: *Apc*-mutation-induced colonic polyposis, azoxymethane-induced formation of colonic aberrant foci and low-dose irradiation combined with diethylstilbestrol-induced mammary tumorigenesis[10,14,138,139]. Although these pre-clinical studies are encouraging and in agreement with genetic studies, conflicting results in some *iNOS*$^{-/-}$ mouse studies have also been reported (TABLE 1). Because of these apparently contradictory findings and the fact that NO mediates multiple normal physiological functions, more detailed evaluation in preclinical models will be required prior to the clinical evaluation of this strategy.

Farnesyltransferase inhibitors
A class of chemical agents that selectively inhibit farnesyltransferase. Farnesyltransferase is responsible for the transfer of a farnesyl group to Ras oncoproteins and other proteins that are involved in signalling for cell transformation and survival, which is usually abnormally active in cancer.

Box 3 | **Role of NO in lymphatic formation, function and metastasis**

In normal tissues, the lymphatic network transports both immune cells and interstitial fluid out of the tissue and therefore maintains immune function and tissue interstitial fluid balance[159]. Growing tumour cells compress lymphatic vessels inside tumours and compromise their function[160]. Conversely, lymphangiogenesis, lymphatic hypertrophy and lymphatic dilation are often found in the peripheral region of the tumour[161]. Tumour cells invade the lymphatic vessels and form metastases within the lymphatic system. Clinical studies indicate that nitric oxide (NO) is involved in this process in several types of tumour. Positive correlation was found between NO synthase (NOS) expression and lymph-node metastasis in patients with head and neck, breast, stomach and gall-bladder tumours (Supplementary information S1 (table)). Recently, NO exposure has been shown to induce vascular endothelial growth factor C (VEGFC) expression in A-431 squamous carcinoma cells and MDA-MB-231 breast cancer cells[162,163]. VEGFC is a putative lymphangiogenic factor that mediates lymphangiogenesis and metastasis[159]. The activity of inducible NOS (iNOS) was positively correlated with VEGFC mRNA expression in patients with lymph-node-positive head and neck tumours[162]. Nitrotyrosine levels were positively correlated with VEGFC immunoreactivity and lymph-node metastasis in patients with invasive breast cancer[163]. These data suggest that NO induces tumour lymphangiogenesis through VEGFC upregulation.

Endothelial NOS (eNOS) might also regulate the formation and/or function of tumour lymphatics[164]. Lymphatic endothelial cells express eNOS in a similar way to blood vascular endothelial cells[165]. VEGFC induces NO production in human umbilical vascular endothelial cells, presumably through VEGF receptor 2 (VEGFR2), which is also expressed in the endothelial cells that line the lymphatic vessels[166]. VEGFC–VEGFR3 signalling activates Akt, which is known to induce eNOS phosphorylation, in these cells[167]. Finally, inhibition of eNOS activity results in reduced VEGFR3-positive cells in tumours[61]. Furthermore, endogenous NO that is produced by eNOS in lymphatic endothelial cells dilates collecting lymphatic vessels and therefore decreases resistance to lymphatic flow in normal tissues[165,168]. Similar regulation of lymph flow might occur in lymphatic vessels associated with tumours. These findings point to the possibility that eNOS in lymphatic endothelial cells mediates lymphangiogenesis, lymphatic hypertrophy, dilation and therefore metastasis. Although no direct evidence has been provided to date, the modulation of NO signalling in lymphatics might provide a unique opportunity to prevent lymphatic metastasis.

Implications and future directions

Evidence is accumulating that NO has an important role in the regulation of tumorigenesis, tumour angiogenesis, vascular functions, progression and metastasis. On the basis of the available literature, one might conclude that, overall, NO promotes *de novo* tumorigenesis when associated with chronic inflammation, angiogenesis and the growth of established solid tumours, whereas it mediates anti-tumour-cell activity against haematogeneously disseminating tumour cells. Unfortunately, there are no preclinical studies yet that show causal relationships between NO production, lymphatic function and metastasis in tumours. However, many compelling histopathological studies demonstrate a positive correlation between NOS expression and/or activity, and lymph-node metastasis (BOX 3). The array of conflicting reports in almost all aspects of NO biology hint at the complexity of the NO signalling network and confound the simple translation of preclinical data from bench to bedside. Recent studies highlight the importance of NO distribution, dose and exposure duration in governing the effects of NO. Therefore, studies of *in vivo* tumours with high spatial and temporal resolution are required to fully understand the role of NO in tumour progression. Owing to experimental challenges, the field currently suffers from a paucity of such data. Development of and progress with *in vivo* experimental techniques, including quantitative imaging of NO, NOS activity and other functional parameters, are beginning to fill these gaps.

Though at an early stage, some preclinical studies that evaluate NOS inhibition as an anti-tumour strategy have provided encouraging results (TABLE 2). Targeting tumour cells for direct cell killing or sensitization to other cytotoxic therapies might require more efficiency and selectivity. Delivery of *iNOS* vectors using a tumour-targeting moiety might strengthen this strategy. For example, iNOS-encoding retroviral vectors that contain a single-chain variable fragmented antibody to carcinoembryonic antigen (CEA) could selectively deliver iNOS to CEA-expressing tumour cells and induce tumour-cell apoptosis[140]. An NO donor could also be used to increase tumour blood flow and oxygenation[89,129]. However, systemic administration of an NO donor lowers blood pressure by dilation of normal arterioles. Tumour perfusion could be decreased by hypotension that is due to a lack of autoregulation in tumour microcirculation. The reduction of nitrite to NO by deoxyhaemoglobin and NO release from *S*-nitrosohaemoglobin preferentially occur at low oxygen tension[141,142]. As tumours are hypoxic, these agents might selectively release NO in tumour vessels. Potentiation of signalling pathways downstream of NO might be an alternative approach. Sildenafil, a phosphodiesterase type-5 inhibitor, enhances cGMP-dependent NO signalling and is used clinically to treat pulmonary hypertension[143]. Sildenafil also significantly increased angiogenesis in a rat focal-ischaemia model[144], which indicates a potential use for the treatment of ischaemic diseases or the improvement of drug delivery to tumours.

Although direct tumour growth-retardation effects are limited, inhibition of NO signalling inhibits tumour angiogenesis and blood flow (TABLES 1,2). It has been shown that the combination of a vascular disrupting agent, combrestatin A-4, and a NOS inhibitor, L-NNA, decreased tumour perfusion more than either agent alone[145]. Combinations of anti-angiogenic agents and NOS inhibitors might also be more effective. Interaction between cyclooxygenase 2 (COX2) signalling and NO signalling is well documented[146,147]. A combination of COX2 inhibitor and iNOS inhibitor has been shown to produce a more comprehensive chemopreventive effect against colon carcinogenesis[14]. In addition, NOS inhibitors have been shown to retard the growth of tumours that are resistant to COX inhibitors[148]. On the other hand, NO-donating, nonsteroidal anti-inflammatory drugs (NSAIDs) showed a more efficient *in vitro* anti-tumour-cell effect and *in vivo* chemoprevention effect than standard NSAIDs[149,150]. NO-donating NSAIDs were originally designed to reduce NSAID toxicity, and the mechanisms of NO delivery from NO-donating NSAIDs and the effect on tumours of NO that is released from NO-donating NSAIDs are not clearly understood. Further studies that elucidate these mechanisms are needed to resolve these potentially conflicting findings.

As discussed in this Review, continuing investigations into the biology of NO will expand our understanding of its many pathological functions and inspire new therapeutic strategies for cancer.

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Competing interests statement
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