Published in final edited form as:

Nat Rev Cancer. 2012 September ; 12(9): 627–637. doi:10.1038/nrc3339.

Aiding and abetting roles of NOX oxidases in cellular transformation

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Abstract

NADPH oxidases of the NADPH oxidase (NOX) family are dedicated reactive oxygen species-generating enzymes that broadly and specifically regulate redox-sensitive signalling pathways that are involved in cancer development and progression. They act at specific cellular membranes and microdomains through the activation of oncogenes and the inactivation of tumour suppressor proteins. In this Review, we discuss primary targets and redox-linked signalling systems that are influenced by NOX-derived ROS, and the biological role of NOX oxidases in the aetiology of cancer.

Cellular transformation is a multistep process that requires an as yet undetermined sequence of genetic alterations and changes in intracellular signalling¹. The hallmarks of cancer, first reviewed by Hanahan and Weinberg in 2000, and updated in 2011, indicate aberrant events that mediate cellular transformation^{2,3}. An underlying event not discussed in these reviews is the role of oxidative stress in these processes. The role of oxidative stress in cellular transformation was first described in 1981 by Oberley and colleagues⁴. This seminal paper described the generation of intracellular hydrogen peroxide, stimulated by insulin, as a second messenger that induces cellular proliferation. Additionally, the authors suggested that increased super-oxide production leads to cellular immortality. This work became known as ‘the free radical theory of cancer’ (REF. 4). Similar to the importance of free radicals, was the discovery of enzymes that are involved in scavenging these radicals, which are referred to as antioxidants⁵. Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the ability of cells to neutralize their reactive intermediates. As early as 1981, when oxidative stress had only been hypothesized to have a role in cellular transformation and the enzymatic sources of ROS were still unknown, it was suggested that enzymes biochemically similar to the NADPH oxidase (NOX) NOX2 (also known as gp91phox) of the human neutrophil were present in nonphagocytic cells^{4,6}. A decade later, other investigators detected high levels of super-oxide and hydrogen peroxide in various cancer cells^{7,8} and confirmed that ROS production could be reduced by diphenyleneiodonium (DPI), a chemical inhibitor of flavoprotein-containing enzymes such as NOX oxidases.

Currently, several sources of ROS in cells and tissues have been identified: the mitochondrial electron transfer chain and NADPH oxidases of the NOX family are the two major sources implicated in cancer. ROS derived from these two major sources are not mutually exclusive, and recent evidence suggests crosstalk exists between these major producers, which is highlighted in this Review. The role of mitochondrial-derived ROS in cancer, produced as a byproduct of mitochondrial respiration, has previously been reviewed^{9–11}. In large part, the biological roles of NOX oxidases in cancer have been identified based on the knowledge gained during examination of the physiological and pathophysiological roles of NOX oxidases in diabetic kidney disease, cardiovascular disease, Alzheimer's disease, fibrosis and atherosclerosis^{12–14}. Although primarily based on *in vitro* data, owing to the early status of determining the function of NOX in cancer, this Review highlights the roles of the NOX complexes in cellular transformation and the maintenance of the malignant phenotype.

The NOX family as a source of ROS in cancer

Since 1999, the discovery and characterization of NOX2 homologues in various cells and tissues has facilitated a better understanding of the molecular mechanisms underlying oxidative stress in cancer. Seven membrane-bound NOX catalytic isoforms, referred to as NOX1 to NOX5, dual oxidase 1 (DUOX1) and DUOX2 have been identified, each of which displays similar but distinct structural, biochemical and subcellular localization characteristics^{13,15–23}. The NOX catalytic subunits that have been implicated in cancer include NOX1, NOX2, NOX4 and NOX5. The roles of DUOX1, DUOX2 and NOX3 in carcinogenesis are not well established and so are not further discussed here. However, it is noteworthy that the DUOX promoters are highly methylated in lung cancer, but the biological importance of this posttranslational modification remains unclear^{24–26}.

NOX-dependent redox signalling occurs at cellular membranes and intracellular structures where the NOX catalytic and regulatory subunits are localized. The NOX catalytic subunits NOX2, NOX1, NOX4 and NOX5 have been detected at the plasma membrane^{27–31}. NOX4 has additionally been detected in the endoplasmic reticulum³², mitochondrial^{27,33,34} and nuclear membranes³⁵. NOX subunits also reside at specific subcellular microdomains such as NOX4 at focal adhesions³⁵, NOX1 at caveoli and lipid rafts^{35,36}, and NOX1 and NOX4 at invadopodia³⁷. With some exceptions, the NOX catalytic subunits have six amino-terminal transmembrane-spanning segments, conserved histidine residues that bind two haems, and carboxy-terminal binding sites for FAD and NADPH. NOX catalytic subunits transfer electrons from NADPH across biological membranes to molecular oxygen. With the exception of NOX5, the facilitation of electron transfer requires the small membrane-bound protein p22phox and cytosolic NOX-regulatory subunits. Most NOX catalytic isoforms use different regulatory subunits. For example, activation of NOX2 oxidase requires p22phox and the recruitment of cytosolic regulatory subunits p47phox, p67phox, p40phox and the GTPase RAC1. On cell stimulation, NOX2 complex formation and activation are mediated by initial phosphorylation and conformational change of the p47phox subunit^{38,39}. p47phox organizes the translocation of the NOX activator subunit p67phox and p40phox to the membrane and docks through its SRC homology domains to the proline-rich region of the p22phox subunit with subsequent recruitment of RAC1. Once the complex is formed, electrons are donated from NADPH and transferred to FAD, reducing it to FADH₂, which is mediated by the activation domain of p67phox. A single electron is then transferred from FADH₂ first iron centre of the NOX catalytic subunit, which can only accept one electron. Oxygen is bound to the second haem centre and receives the electron from the inner first haem. One-electron reduction of molecular oxygen (O₂) generates superoxide anion (O₂^{•-}), which is then dismutated to hydrogen peroxide (H₂O₂), either spontaneously at an acid pH or catalytically by superoxide dismutase (SOD). For NOX4, hydrogen peroxide

rather than superoxide is the predominant species detected by standard molecular reagents. As there is no thermo-dynamically viable mechanism for a haemcatalysed two-electron reduction of oxygen, the predominant detection of hydrogen peroxide generation is probably the result of the rapid kinetics of dismutation and/or due to a conserved histidine residue located in the extended E-loop of the NOX4 molecule, which may serve as a source of protons or as a trapping mechanism for the spontaneous dismutation of superoxide at this site^{13,30,40,41}.

Reconstitution assays and the characterization of NOX isoforms show that p47phox and p67phox do not robustly activate other NOX isoforms in non-phagocytic cells. This led to the identification and cloning of human homologues of p47phox (also known as NOXO1) and p67phox (also known as NOXA1)^{15,42}. Although the mechanisms of activation for NOX1 and NOX3 are similar to those of NOX2, NOX1 interacts with NOXO1 and NOXA1 in addition to tyrosine kinase substrate 4 (TKS4; also known as SH3PXD2B) and RAC1 on activation^{13,19,21,37,42–46}. By contrast, the isoforms NOX4 and NOX5 do not require any of the classic cytosolic subunits¹³. In some circumstances, NOX4 interacts with proteins that enhance its activity, namely DNA polymerase- δ -interacting protein 2 (POLDIP2) and tyrosine kinase substrate 5 (TKS5; also known as SH3PXD2A)^{37,47,48}. NOX4 seems to be a constitutively active enzyme that is primarily regulated at the level of its expression in response to various stimuli¹³, and NOX5 has N-terminal cytoplasmic EF-hand calcium-binding motifs that bind calcium for activation. A summary of the NOX catalytic subunits with their regulatory subunits (NOX complexes) is shown in FIG. 1. The structure and function of individual NOX oxidase subunits have been comprehensively reviewed^{13,19,21}.

NOX catalytic and regulatory subunits have been implicated in one or more of the most common cancer types. Increased mRNA and/or protein expression of NOX1, NOX2, NOX4 and NOX5 or their regulatory components has been detected at higher levels in various cultured cancer cell lines^{34,49–61} or human tumours^{34,55,58,59,61–75} compared with normal controls at early and late stages of tumorigenesis, suggesting that ROS derived from NADPH oxidases may be important in both the initiation and the maintenance phases of tumour development (TABLE 1).

Biological roles of NOX-derived ROS in cancer

There are two biological outcomes in response to the exposure of cells to ROS. ROS are chemically reactive and can directly damage biomolecules such as nucleic acids, resulting in genomic instability. ROS can also directly or indirectly activate or inactivate redox-sensitive signalling pathways, damage lipids and modify proteins to alter cellular functions, promoting autonomous cell growth and immortality. Therefore, a fundamental question is whether NOX-derived ROS have a role in genomic instability or a biochemical role in the initiation and maintenance of cellular transformation? We address this question by examining the biological and molecular bases of NOX-derived ROS actions in the principal hallmarks of cancer (FIG. 2).

NOX-derived ROS and genomic instability

Defining direct roles for NOX-derived ROS in mutagenesis and genomic instability is complex when considering putative interactions between other intracellular reactive species. In general, superoxide is converted into hydrogen peroxide enzymatically by the cytosolic antioxidant superoxide dismutase 1 (SOD1) or the mitochondrial SOD2 and is then converted to water by glutathione peroxidase or catalase. However, this conversion is not 100% efficient and in some conditions SOD expression and activity can be altered. SOD1 activity is reduced in human renal cancer tissue⁷⁶, and SOD1 and SOD2 expression are reduced in prostate cancer tissue⁷⁷, whereas SOD1 expression is increased in breast cancer

tissue⁷⁸. Alterations of SOD expression can be a 'double-edged sword'. For example, overexpression of SOD1, as is the case in breast cancer, will theoretically generate high levels of hydrogen peroxide, capable of diffusing across biological membranes and constitutively activating redox-signalling pathways. Low expression of SOD1 promotes interactions of superoxide, which cannot cross biological membranes⁷⁹, with other cellular reactive species such as nitric oxide (NO) to produce peroxynitrite (OONO⁻) or hydrogen peroxide (H₂O₂) to form a hydroxyl radical (•OH), which are both highly reactive intermediates that can quickly damage surrounding macromolecules such as nucleic acids, lipids and proteins. Oxidation of nucleic acids results in the formation of adducts with deoxyguanine (8-OH-dG), which if not repaired can potentially generate mutations⁸⁰. Mitochondrial DNA is highly susceptible to damage because it is not protected by histones and is therefore directly exposed to ROS. In addition, DNA repair capacity is less efficient in the mitochondria¹⁰. The recent finding that NOX4 localizes to the mitochondria^{27,34} suggests that NOX oxidases might mediate mitochondrial DNA damage. Moreover, there is a growing body of evidence linking mitochondrial metabolism to carcinogenesis. Notably, NOX4-derived ROS can directly oxidize mitochondrial complex subunits, leading to mitochondrial dysfunction^{33,81}. The identification of a superoxide-generating NOX4 in the nucleus raises the possibility of direct alteration of nuclear DNA and proteins by the oxidase⁸². Overexpression of HRAS V12 in normal thyroid cells induces NOX4-derived ROS, DNA replication rate and DNA damage, leading to cellular senescence. Silencing of NOX4 or the treatment of cells with the antioxidant *N*-acetyl-cysteine (NAC) both reduce RAS-mediated DNA lesions, DNA foci formation and cell senescence⁸³. Although the mechanisms have not been elucidated, NOX4-mediated DNA lesions in cells expressing oncogenic HRAS may be a result of increased DNA replication rate. As RAS signalling is activated in a number of cancers, which do not undergo cellular senescence, NOX4 may have a subsequent role in nuclear genomic hits and instability in these cancers.

Taken together, the subcellular localization where reactive species are generated, interactions between reactive species, expression of antioxidants present in the milieu and functional DNA repair pathways all have roles and can mediate outcomes of mutagenesis and genomic instability.

Role of NOX oxidases in autonomous cell growth and survival

In general, the roles and mechanisms by which NOX oxidases mediate autonomous cell growth and cell survival remain unclear and require further investigation. However, there are some initial studies that have provided insight into the function of NOX oxidases in these processes. Before the cloning of NOX isoforms and their identification in somatic cells, it was suggested that superoxide and hydrogen peroxide function as mitogenic stimuli through biochemical processes that are common to growth factors. The addition of low concentrations of superoxide or hydrogen peroxide to cultured cells, including fibroblasts, epidermal cells, and leukaemic and osteoblastic cells, stimulates growth and induces the expression of immediate early genes, such as *FOS* and *JUN*^{84–86}. Conversely, antioxidants that neutralize hydrogen peroxide inhibit tyrosine kinase-dependent signalling^{87–89}.

NOX1-, NOX2- and NOX4-derived ROS mediate cell proliferation of normal cultured cells in response to various growth stimuli that activate receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR)^{90–93}, suggesting that the mitogenic effects of NOX-mediated ROS in cancer cells with aberrant growth factor signalling may be a universal occurrence. NOX-derived ROS promote growth factor-induced tyrosine autophosphorylation predominantly by inactivating protein tyrosine phosphatases (PTPs), resulting in hyperphosphorylation and activation of the RTK and downstream signalling molecules^{88,94,95}. Nucleophilic amino acids, including the sulphur-containing cysteine residues, are highly susceptible to redox modification⁹⁶. The

catalytic domains of PTPs contain redox-sensitive cysteine residues that undergo oxidative modification, which is predominantly mediated by hydrogen peroxide, resulting in impaired catalytic activity^{97–100}. The modification of nucleophile amino acid residues is determined and surrounding amino by a characteristically low pK_a acids, as well as its location in the tertiary structure of a protein making it susceptible or not to redox modification^{96,101}. The oxidation of cysteine residues can be mild and reversible (by intracellular-reducing agents) or severe and irreversible. In-gel phosphatase activity assays suggest that NOX-derived ROS selectively target intracellular PTPs rather than nonspecifically oxidize PTPs, with the specificity of PTP oxidation probably attributed to the proximity of the NOX-derived ROS to the phosphatase^{101–104}. To date, phosphatases that are known to be regulated by NOX oxidases include the tyrosine phosphatases PTP1B, PTEN, SRC homology region 2 domain-containing phosphatase 1 (SHP1), SHP2, PTP-PEST, CD45, low-molecular-weight protein tyrosine phosphatase (LMW-PTP) and the serine/threonine phosphatase PP1 (REFS 105,106). This list is likely to grow, as protein phosphatases are an abundant group of proteins in mammalian cells¹⁰⁷. Constitutive NOX-derived ROS that are produced in hepatoma and epidermoid carcinoma cells potentiate autonomous cell growth through the inactivation of PTP1B¹⁰¹. Alternatively, in liver tumour cells, NOX1 regulates autocrine cell growth by regulating mRNA expression of *EGFR* and one of its ligands, transforming growth factor- α (*TGF α*), through redox-dependent activation of p38MAPK and AKT signalling pathways¹⁰⁸.

Inactivation of phosphatases by NOX-derived ROS is linked to cell survival, in large part through the regulation of conventional survival pathways such as Janus kinase (JAK)–signal transducer and activator of transcription (STAT), AKT, nuclear factor- κ B (NF- κ B) and p53 signalling. In pancreatic cancer cells, insulin-like growth factor 1 (IGF1) or serum can activate the anti-apoptotic JAK2–STAT pathway and AKT, through NOX4-mediated inhibition of LMW-PTP and PP1 phosphatases, respectively^{98,109}. Whereas inducible expression of BCR–ABL in chronic myelogenous leukaemia cells enhances NOX4-dependent cell survival through inactivation of PP1 and upregulation of the PI3K–AKT survival pathway¹¹⁰. In other studies, the activation of the G protein-coupled receptor (GPCR) leukotriene receptor BLT2 leads to cell survival through the induction of NOX1- and NOX4-derived ROS production through an ERK- and AKT-dependent mechanism in cultured bladder and breast cancer cell lines (REFS 52,111). NOX1- and NOX4-derived ROS promote cell survival in colon cancer cells and melanoma cells, respectively, through a putative NF- κ B mechanism^{51,67}, and inhibition of AKT by NOX4-derived ROS promotes cell survival in cultured pancreatic cancer cells^{60,112}.

More recently, a novel link between NOX activation and inactivation of the pro-apoptotic tumour suppressor protein p53 has been described in cancer cells. NOX1 mediates cell survival through sirtuin 1 (SIRT1) activation, which causes deacetylation and inactivation of p53, ultimately impairing p53-mediated transcriptional induction of pro-apoptotic genes^{113–115}. The putative link between NOX and SIRT1 activity could be important for many other biological outcomes, as SIRT deacetylates a broad range of protein substrates and has been implicated in chemotherapy resistance¹¹⁶ and in the regulation of FOXO-dependent expression of glucogenesis-promoting genes in cancer¹¹⁷. The link between NOX and deacetylation needs further exploration.

The small GTPase RAS has a key role in the establishment of growth autonomy. In human colorectal tumours, a positive correlation of NOX1 expression with *KRAS* mutations has been described¹¹⁸ but this is not the case in prostate cancer in which NOX1 expression is high and *KRAS* mutations are absent or very infrequent⁷². Approximately 30% of all human cancers harbour oncogenic mutations in one of the RAS genes, giving rise to constitutively activated RAS signalling. Independent of RAS mutations, RAS can be directly activated by

oxidation on susceptible cysteine residues within the molecule; however, a direct role for NOX-dependent activation of RAS has not been studied in cancer^{100,119,120}. Activation of RAS promotes NOX-dependent oxidative stress in cancer cells. Overexpression of KRAS in several cell types increases the transcription of NOX1 through RAF–MEK–ERK-dependent phosphorylation of the transcription factor GATA6 with subsequent binding to the *NOX1* promoter^{121,122}. NOX1-dependent ROS generation is required for RAS-induced anchorage-independent cell growth and tumour formation in athymic mice¹²². In a subset of RAS-transformed cells, NOX1 may potentiate cell proliferation and anchorage-independent cell growth through the cooperation of RAS–MEK and canonical WNT– β -catenin-dependent signalling^{123,124}. Binding of WNT– β -catenin to its receptor dissociates the axin-binding protein Dishevelled (DVL) from nucleoredoxin (NRX), resulting in DVL-dependent stabilization of β -catenin. Stabilized β -catenin migrates into the nuclei and associates with transcription factor T cell factor (TCF)/LEF to activate cell cycle and proliferation-controlling genes such as cyclin D1 and *MYC*. Overexpression of NRX inhibits WNT-induced cell proliferation in RAS-transformed cells, which is reversed by co-expression of NOX1 (REF. 123). NOX1-dependent activation of WNT– β -catenin-mediated cell growth occurs through redox targeting of NRX with the disruption of NRX–DVL complexes¹²³. More recently, Du and colleagues demonstrated that superoxide, produced by NOX2, mediates pancreatic cancer cell growth in *KRAS*-transformed pancreatic ductal epithelial cell lines, through as yet unidentified mechanisms¹²⁵.

Maintaining cell growth is necessary for cancer cell proliferation and survival. The PI3K–AKT–mTOR signalling pathway induces many responses in cells, including the maintenance of mRNA translation, cell survival and metabolism, and is activated in the majority of cancers^{126–131}. The tumour suppressor PTEN, a dual lipid and tyrosine phosphatase, inhibits PI3K signalling and is mutated or post-translationally inactivated in cancer. As with most other PTPs, PTEN is susceptible to inhibition by ROS-mediated cysteine oxidation¹³². In normal cells that are stimulated with growth factors, PTEN has been identified as a primary target of NOX1-derived hydrogen peroxide; however, a role for NOX4-derived ROS has also been implicated in PTEN inactivation^{133–136}. Although these studies were conducted in normal cells that were stimulated with growth factors, the overall observation is highly relevant for cancer, as inactivation of PTEN results in increased PI3K-dependent production of phosphatidylinositol-3,4,5-trisphosphate and activation of AKT by phosphoinositide-dependent 1 (PDK1) at Thr308 and the mTORC2 complex at Ser473 (REFS 133,137–139). p22phox-based NOX-derived ROS production is enhanced in von Hippel–Lindau (VHL)-deficient renal carcinoma cells (RCC). Gene silencing of p22phox-based NOX oxidases in VHL-deficient cells inhibits phosphorylation of AKT on its mTORC2-dependent site, Ser473, suggesting a novel role of NOX oxidases in the regulation of mTORC2, through as yet unidentified mechanisms^{27,49}. In addition, p22phox, probably through its interaction with NOX4 and NOX1, indirectly activates mTORC1 signalling downstream of PTEN through redox inactivation of tuberlin in RCC cells. The tuberlin–hamartin hetero-dimer is a key negative upstream regulator of mTORC1 signalling. When tuberlin is phosphorylated by AKT on Thr1462 this results in its dissociation from hamartin with subsequent ubiquitin-mediated degradation¹⁴⁰. Treatment of VHL-deficient cells with DPI or downregulation of p22phox results in decreased tuberlin phosphorylation on Thr1462 and stabilizes tuberlin protein levels²⁷. Inactivation of tuberlin by p22phox-based NOX oxidases results in mTORC1-dependent phosphorylation of initiation factor 4E-binding protein 1 (4EBP1) and ribosomal protein S6 kinase 1 (S6K), which are required for the stimulation of cap-dependent translation of key target genes that are involved in tumorigenesis^{27,49,141}.

Glucose uptake is crucial for cancer cell growth and survival. Glucose transporters are upregulated in cancer cells and enhance glucose uptake. There are various mechanisms by

which NOX oxidases facilitate glucose uptake, most of which have been studied in renal cancer cells. The VHL tumour suppressor protein is inactivated in ~70–80% of clear cell RCC cells. VHL is the substrate recognition subunit of a multimeric E3 ubiquitin ligase complex, which under normal oxygen conditions targets the α -subunits of the hypoxia-inducible factors (HIF α) for regulated protein degradation^{142–144}. Post-translational hydroxylation of the HIF α subunits, by proline hydroxylases, is necessary for binding of the VHL–E3 complex¹⁴⁵. In the absence of VHL, HIF α subunits are stabilized. However, in VHL-deficient RCC cells, maintaining HIF2 α expression requires ongoing mRNA translation, as treatment of VHL-deficient cells with cyclohexamide, a general translation inhibitor, reduces HIF α protein expression⁴⁹. NOX1- and NOX4-derived ROS levels are increased in VHL-deficient cells and maintain HIF2 α expression through an AKT-dependent mRNA translational mechanism^{27,49,146}. Importantly, HIF2 α is a master transcriptional regulator of genes that are involved in cell growth, survival and angiogenesis, suggesting an indirect role for NOX oxidases in the aforementioned biological processes. For example, HIF2 α is the transcriptional regulator of the renal mitogen TGF α , suggesting an indirect role of NOX-derived ROS in RCC autonomous cell growth. Consistent with this, treatment of VHL-deficient RCC cells with the flavoprotein inhibitor DPI reduces HIF2 α expression and cell growth⁴⁹. Similarly, HIF2 α regulates expression of glucose transporter 1 in RCC cells, suggesting a role for p22phox-based NOX oxidases in glucose uptake in VHL-deficient cells. Conversely, stabilization of HIF α -dependent glucose uptake in cancer cells that are VHL competent relies on mechanisms that inhibit proline hydroxylation. For example, fumarate hydratase (FH)-deficient renal cancer cells proliferate rapidly and require HIF1 α -dependent glucose uptake for survival^{147,148}. HIF1 α is stabilized in FH-deficient cells, in spite of VHL expression, through p47phox-dependent NOX oxidase- and fumarate-dependent inhibition of proline hydroxylation¹⁴⁸.

In leukaemic cells, however, NOX2- and NOX4- derived ROS promote glucose uptake, cell survival and proliferation through putative mechanisms involving SRC activation and translocation of GLUT1-containing vesicles, which is independent of HIF^{57,149}.

Role of NOX-derived ROS in angiogenesis

Tumour angio-genesis is necessary for vascularizing solid tumours in order to provide nutrients and oxygen for growth past a small diameter of ~1–2 mm. New blood vessel formation, arising from the existing vasculature, requires angiogenic growth factors (such as vascular endothelial growth factor (VEGF), which activates matrix metal-loproteinases (MMPs)), the proliferation of endothelial cells and the formation of blood vessel tubules. VEGF is a target for therapeutic angiogenesis in many cancers, and evidence suggests that compartmentalized NOX-derived ROS coordinate the angiogenic switch by enabling increased VEGF production by tumour cells. This propagates VEGF autocrine (for survival and migration) and paracrine (for tumour angiogenesis) signalling^{106,150}. These processes are initiated when a tumour experiences hypoxia. Human *NOX4* and *NOX2* promoters harbour putative hypoxia-responsive elements (HREs) to which HIFs bind under hypoxic conditions and which thus may be characterized as oxygen sensors that are activated when solid tumours experience low oxygen tension. Indeed, *NOX4* and *NOX2* mRNAs are upregulated *in vitro* and *in vivo* in response to stabilization of HIFs, whereas NOX1-based oxidase complexes are post-translationally activated under hypoxic conditions^{151–155}. NOX1- and NOX4-generated ROS increase *VEGF* and *VEGFR* mRNA levels and other markers of the angiogenic switch, thereby promoting vascularization and rapid expansion of malignant melanomas, prostate cancer and ovarian cancer cells in chicken chorioallantoic membranes or *in vivo* xenograft mouse models^{72,75,156,157}. In ovarian cancer cells, NOX4-derived ROS and mitochondrial-derived ROS were found to regulate VEGF levels through HIF1 α expression, and both sources of ROS were found to be required for tumour-induced

angiogenesis and tumour growth⁷⁵. NOX-dependent upregulation of *VEGF* mRNA expression can also be independent of HIF α . In RAS-transformed colon cancer cells, silencing of NOX1 using small inhibitory RNAs, inhibits ERK-dependent phosphorylation of the transcription factor SP1, thereby inhibiting SP1-dependent upregulation of *VEGF* mRNA and neovascularization in nude mice¹⁵⁸. The subcutaneous injection of melanoma cells or lung carcinoma cells in *NOX1*-deficient mice or in wild-type mice treated with GKT136901 (an inhibitor of NOX1 and NOX4) reduces tumour angiogenesis through AKT-dependent suppression of the anti-inflammatory nuclear hormone receptor peroxisome-proliferator activated receptor- α (PPAR α), a negative regulator of nuclear factor- κ B (NF- κ B) signalling¹⁵⁹. siRNA-mediated downregulation of NOX1 in cultured melanoma or lung carcinoma cells inhibits NF- κ B activation of VEGF and MMPs¹⁵⁹.

Paracrine activation of VEGFR2 in endothelial cells by VEGF that is secreted by tumour cells is initiated at caveoli and lipid rafts by NOX2-dependent complexes. In addition, biological events, such as proliferation, migration and tube formation, possibly involve the generation of ROS by NOX1- and NOX4-dependent complexes at sites of focal adhesions and membrane ruffles in endothelial cells¹⁰⁶.

A role for NOX oxidases in cell invasion and metastasis

Tumour metastasis is a multistep process that requires extracellular remodelling and intracellular changes such as epithelial–mesenchymal transition (EMT), reduced cell adhesion and increased migration, as well as degradation of the extracellular matrix. The tumour environment is complex, with cancer cells surrounded by various other cell types, including stromal cells, endothelial cells and inflammatory cells, which secrete high levels of growth factors, such as TGF β , and cytokines, such as interleukins, into the extracellular fluid. NOX oxidases are responsive to soluble mediators that are present in the extracellular fluid, which influence the stimulation of cell migration and invasion. Stromal cells secrete TGF β when co-cultured with breast cancer epithelial cells, stimulating NOX4-dependent migration of epithelial cells¹⁶⁰. However, tumour-associated macrophages (TAMs) secrete interleukin-1 β (IL-1 β) when co-cultured with breast cancer epithelial cells, stimulating NOX-derived ROS with subsequent activation of the SRC–MAPK–AP1 pathway in breast cancer cells, leading to the transcriptional upregulation of cyclooxygenase 2 (COX2) levels, which is associated with increased proliferation and the metastasis of breast cancer cells¹⁶¹. A more recent finding suggests that NOX4 has a role in cytokine production in renal cancer cells that are exposed to hypoxia¹⁶². Renal cancer cells, but not normal kidney cells, exposed to hypoxia secrete IL-6 and IL-8 in a NOX4-dependent manner. In turn, the exposure of renal cancer cells to IL-6 and IL-8 induces migration and invasion¹⁶².

Growth factors and cytokines that are present in the tumour milieu can alternatively negatively influence the antitumour ability of immune effector cells, such as T cells, in part through the expansion of myeloid-derived suppressor cells (MDSCs). MDSCs are immature myeloid progenitor cells that suppress T cell function in cancer. Interestingly, NOX2 is upregulated in a STAT3-dependent manner and hyperactivated in MDSCs in a variety of tumour models and human MDSCs¹⁶³. MDSCs that were isolated from the spleens of EL-4 tumour-bearing NOX2-deficient mice failed to suppress T cell function and resulted in differentiated MDSC cells; however, the lack of NOX2 did not result in tumour rejection¹⁶³. Taken together, and owing to the fact that NOX2 is highly expressed in infiltrating immune cells, these data suggest novel roles for NOX2 in cancer immunology, which should be further explored.

Agonists that enhance metastasis induce structural intracellular changes in tumour cells, such as the formation of invasive microdomains called invadopodia¹⁶⁴. NOX1 and NOX4 are present in invadopodia, and NOX4 also localizes to focal adhesions^{35,106,164–166}. The

subcellular localization of NOX1 and NOX4 to these membrane domains allows the formation of active redox signalling platforms that concentrate NOX-derived ROS in a small area, allowing them to coordinate cell adhesion, migration and invasive matrix degradation^{35,106,165}. SRC and the SRC family of kinases are also located in invadopodia or in focal adhesions and are crucial for the invasiveness and metastatic potential characteristic of some cancer cells^{164,166–170}. SRC proteins can function as both upstream modulators and downstream effectors of NOX oxidases^{108,123,171}. SRC contributes to the activation of NOX2 and NOX1 through phosphorylation of p47phox and NOXA1, respectively, or through the activation of the small GTPase RAC1, leading to the formation of an active NOX enzymatic complex^{123,171–173}. Moreover, TKS4 and TKS5 are phosphorylated by SRC during NOX activation, which is necessary for invadopodia formation and NOX1- and NOX4-derived ROS generation at this microdomain^{37,58,164,174,175}. Note that p22phox also seems to interact with TKS proteins in the NOX complex³⁷. SRC is also reported to be a downstream target of NOX1-, NOX2- and NOX4-derived ROS^{108,176,177}. NOX-dependent SRC regulation is predominantly mediated through the control of its tyrosine phosphorylation, which is regulated by PTP-PEST that is also present in invadopodia^{165,168}.

Other factors, such as MMPs, are present at sites of invadopodia. The activation of MMPs by hydrogen peroxide has been demonstrated in several cancers and may be partly dependent on an RTK–PI3K–NF- κ B signalling pathway^{178–180}. Proteolytic degradation of the extracellular matrix has been shown to involve NOX-dependent upregulation of MMP expression and activity¹⁸¹. Binding of leukotriene B4 to BLT2 elicits NOX4-dependent ROS generation that mediates STAT3- and MMP2-dependent invasiveness and metastasis of ovarian cancer cells¹⁸². In RAS-transformed cells, the BLT2–NOX1–NF- κ B–MMP9 cascade was also reported to be important for tumour cell invasion and metastasis^{183–185}. Additionally, NOX1-derived ROS, NF- κ B and MMP9 are required for the motility of KRAS-transformed normal kidney cells and for the EGF-mediated migration of colon cancer cells through NOX1-dependent inhibition of LMW-PTP, thus blocking RHO activity, a key regulator of cytoskeletal contractility^{186,187}.

Taken together, NOX oxidases coordinate a number of biological responses that are involved in cellular invasion and metastasis through intimate redox regulation of SRC and NF- κ B signalling at discrete subcellular sites, such as invadopodia, and diffusely through moderating communication in the tumour milieu.

Perspectives

NADPH oxidase complexes regulate broad, but specific, redox-signalling pathways in various cell types through the discrete localization of NOX-derived ROS to the plasma membrane, mitochondria, endosomes, focal adhesions, invadopodia, caveolae, endoplasmic reticulum and nuclei, which together coordinate the activation of oncogenes or the inactivation of tumour suppressor proteins that are involved in tumour cell growth and survival, angiogenesis and cell invasion. A summary of oncogenes activated by, and tumour suppressor proteins inactivated by, NOX-derived ROS in these processes is depicted in FIG. 2.

It should be emphasized that this area of cancer research is still in its infancy and the biochemical roles for specific NOX complexes in cancer that are relevant to DNA repair, cellular metabolism and the Warburg effect, microRNA regulation, cancer immunology and cancer stem cell biology remain unexplored. Much like the discovery of kinases and phosphatases, NOX-dependent redox post-translational modifications may represent the next major advances in cell and cancer biology. Identification of NOX target proteins should move the field forwards. The lack of available NOX knockout and transgenic animal models

has hindered and will continue to hinder our understanding of the role of NOX oxidases in cancer. It is noteworthy that the gene encoding NOX5 is absent in the mouse genome.

In summary, most of the studies discussed above are predominantly based on *in vitro* work, and so fail to address the biological complexity that exists in tumours, such as intratumour heterogeneity, microenvironmental changes and the systemic influence of factors, including growth factor levels, inflammation and nutrition. In these changing environments, some cells will gain function while others that have intact apoptotic pathways will undergo necrosis or apoptosis. As outlined in FIG. 3, we hypothesize that, owing to the pleio-tropic nature of NOX-derived ROS, tumour cells that upregulate NOX oxidases and NOX-derived ROS will progress and those that do not will necrose, apoptose or exhibit a biochemically benign phenotype. As the tumour progresses, NOX-derived ROS will maintain activated growth pathways, evade cell death and initiate angiogenesis and metastasis.

Acknowledgments

The authors acknowledge H. E. Abboud, R. A. Clark and R. Li for critical reading of the Review and F. Wauquier for unconditional assistance. Supported by Veterans Administration Career Development Award, NIH R01 NCI CA131272 (K.B.).

Glossary

Focal adhesions	Specific types of large macromolecular assemblies through which both mechanical force and regulatory signals are transmitted
Invadopodia	Protrusions of the plasma membrane that contain adhesive proteins and proteolytic enzymes
Dismutation	A specific type of redox reaction in which a species is simultaneously reduced and oxidized to form two different products, in this case molecular oxygen and hydrogen peroxide

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At a glance

- As early as 1981, it was suggested that enzymes biochemically similar to the NADPH oxidase (NOX) NOX2 were present in all cell types, not just in neutrophils. A decade later, high levels of superoxide and hydrogen peroxide were found in various cancer cells, and these levels could be reduced by diphenyleneiodonium (DPI), a chemical inhibitor of flavoprotein-containing enzymes, such as NOX oxidases.
- The mitochondrial electron transfer chain and NADPH oxidases of the NOX family are the two major sources of reactive oxygen species (ROS) that are implicated in cancer.
- Seven membrane-bound NOX catalytic isoforms (NOX1–5) and dual oxidase 1 (DUOX1) and DUOX2 have been identified, each of which displays similar but distinct structural, biochemical and subcellular localization characteristics.
- NOX-dependent redox signalling occurs at cellular membranes and intracellular structures where the NOX catalytic and regulatory subunits are localized. The NOX catalytic subunits NOX2, NOX1, NOX4 and NOX5 have been detected in the plasma membrane. NOX4 has additionally been detected in the endoplasmic reticulum, mitochondrial and nuclear membranes. NOX subunits also reside at specific subcellular microdomains, such as NOX4 at focal adhesions, NOX1 at caveoli and lipid rafts, and NOX1 and NOX4 at invadopodia.
- NOX catalytic and regulatory subunits have been implicated in one or more of the most common cancer types. Increased mRNA and/or protein expression of NOX1, NOX2, NOX4 and NOX5 or their regulatory components have been detected at higher levels in various cultured cancer cell lines or human tumours compared with normal controls at early and late stages of tumorigenesis, suggesting that ROS derived from NADPH oxidases may be important in both the initiation and the maintenance phases of tumour development.
- Initial studies indicate that NOX oxidases can effect several of the hallmarks of cancer, including genomic instability, autonomous growth and survival, angiogenesis, invasion and metastasis.

Figure 1. Structure and molecular organization of the NADPH oxidases of the NOX family
 Part **a** shows the topology and the enzymatic reaction catalysed by the NADPH oxidase (NOX) enzymes. Parts **b–e** represent the molecular structure of NOX2, NOX1, NOX4 and NOX5, which are predominantly expressed in carcinoma cells. All NOX proteins can form a complex with p22phox, but the cytosolic subunits differ between the NOX oxidase isoforms. e, electron; FAD, flavin, superoxide; POLDIP2, DNA polymerase- δ -interacting adenine dinucleotide; H, Haem; H_2O_2 , hydrogen peroxide; O_2 protein 2; TKS, tyrosine kinase substrate.

Figure 2. Integration of NOX oxidase-derived ROS with the hallmarks of cancer

This figure shows the potential signalling pathways that are activated (+) or inactivated (–) by NADPH oxidase (NOX) oxidases and how they relate to some of the suggested hallmarks of cancer cells, such as deregulated cell growth and angiogenesis. GPCRs, G protein-coupled receptors; HIFs, hypoxia-inducible factors; MMPs, matrix metalloproteinases; PPase, protein phosphatase; PTPs, protein tyrosine phosphatases; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; TSC2, tuberous sclerosis 2; VEGF, vascular endothelial growth factor.

Figure 3. A hypothesis for the function of NOX-derived ROS in the progression of carcinogenesis Multiple genetic and epigenetic alterations (HITS) are required for cellular transformation. The cells that upregulate NADPH oxidase (NOX) oxidases and NOX-derived reactive oxygen species (ROS) will maintain unregulated cell growth, evade cell death and progress towards the development of a clinically relevant tumour. Cells that do not activate NOX-derived ROS and that have intact apoptotic pathways will undergo apoptosis or necrosis. In tumours of approximately ~1–2 mm in diameter, NOX oxidases sense hypoxia and can help to mediate activation of the angiogenic switch while maintaining unregulated cell growth and evasion of cell death. As the tumour continues to grow, a complex tumour environment, including stromal cells, endothelial cells and tumour-associated macrophages (TAMs), secrete various metastatic agonists. NOX oxidases are responsive to the extracellular fluid surrounding the tumour environment and facilitate invasion and metastasis.

Table 1

NOX oxidase subunit expression in common cancer types

Cancer type	NADPH oxidase subunit	Refs
Bladder	p67phox	53
	NOX4	59
	NOX1	73
	NOX4	34
Breast	NOX1	52,65
	NOX5	55
	RAC1	66,68
	TKS5	58
Colon and rectal	NOX4	63
	NOX1	55,67
	NOXO1 and NOXA1	55
	RAC1	66,68
Kidney	NOX4	49,64
	NOX1	64
	p22phox	49,64
Leukaemia	NOX2 and NOX4	55,57
	NOX5	70
Lung	RAC1	68
Melanoma	NOX4	55,61
	NOX2, p22phox, p47phox and p67phox	51
	NOX5	55
Non-Hodgkin's lymphoma	p22phox	69,71
Pancreatic	NOX4 and p22phox	60
Prostatic	NOX4	56
	NOX1	62,72
	NOX2	56
	NOX5	50,54
	p22phox	56
Thyroid	NOX4 and p22phox	74
Ovarian	NOX4	55,75
	NOX1	55,65