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Michael Y. Bonner, *Emory University*
Jack Arbiser, *Emory University*

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Targeting NADPH Oxidases for the Treatment of Cancer and Inflammation

Michael Y. Bonner and **Jack L Arbiser****

Departments of Dermatology*, Emory University School of Medicine, Atlanta, GA, Atlanta Veterans Administration Medical Center, Atlanta, GA

Abstract

NADPH oxidases are a family of oxidases that utilize molecular oxygen to generate hydrogen peroxide and superoxide, thus indicating physiological functions of these Highly reactive and short lived species. The regulation of these NADPH oxidases (nox) enzymes is complex, with many members of this family exhibiting complexity in subunit composition, cellular location, and tissue specific expression. While the complexity of the nox family (Nox1–5, Duox1,2) is daunting, the complexity also allows for targeting of NADPH oxidases in disease states. This review will discuss which inflammatory and malignant disorders can be targeted by nox inhibitors, as well as clinical experience in the use of nox inhibitors.

Introduction

NADPH oxidases are a diverse family of enzymes that give rise to superoxide and hydrogen peroxide, cellular second messengers that play critical roles in human pathology and pathophysiology. While the human role of NADPH oxidases was first elucidated in a deficiency syndrome, chronic granulomatous disease, current information implicates excessive generation of reactive oxygen as a more important mechanism for inflammation and carcinogenesis. Failure to downregulate reactive oxygen generation leads to persistent inflammation in virtually all organ systems. Second, persistent reactive oxygen generation is also carcinogenic, leading to a distinct tumor phenotype known as the reactive oxygen driven tumor. Recognition of this phenotype is important for the following reasons. First, it occurs in all organ systems and can be recognized epidemiologically and histologically. Second, tumors caused by reactive oxygen survive based upon reactive oxygen driven signaling systems. The implications of these findings are that these tumors can be both prevented and treated by reactive oxygen inhibitors. Similarly, inflammatory disorders can be treated by reactive oxygen inhibitors, and this may lead to prevention of neoplasms secondary to persistent inflammation. Thus, both common inflammatory and tumor systems adapt to inflammatory stress by adopting the signaling pathways involved in chronic inflammation, especially reactive oxygen driven NFkB activation.

**Corresponding author. Jack L Arbiser, Department of Dermatology, Emory University School of Medicine, 1639 Pierce Drive, WMB 5309, Atlanta, GA 30322, Tel (404) 727-5063, Fax (404) 727-5897, jarbise@emory.edu.

Identification of the Reactive Oxygen Driven Phenotype

Initial identification of the role of reactive oxygen in neoplasia came from the pioneering studies of Warburg, who identified the fact that advanced tumors used glycolysis as a method of generating ATP, even in the presence of adequate oxygen, and the studies of Carl Nathan, who observed high levels of reactive oxygen in melanoma cells (Warburg, 1956) (Szatrowski and Nathan, 1991). The Lambeth group identified additional members of the nox family, and demonstrated that overexpression of nox1 could contribute to angiogenesis and tumorigenesis (Arbiser et al., 2002b). Our interest in reactive oxygen stemmed from the different behavior of the three forms of endothelial neoplasms observed in humans. Hemangioma, which is the most common, is characterized by rapid growth, followed by regression (Takahashi et al., 1994). Given the unique ability of hemangiomas to regress, we hypothesized that hemangiomas could be driven by reactive oxygen, and sudden loss of reactive oxygen could mediate regression. Thus, hemangiomas could be an example of a reactive oxygen driven tumor. Vascular malformations are characterized by lack of regression, but continued growth with the growth of the patient (Takahashi et al., 1994). Angiosarcoma, a malignant endothelial tumor, is characterized by defects in p53, especially mutant p53, and death due to invasion and distant metastasis (Zietz et al., 1998). We hypothesized that the distinct clinical behavior of these similarly appearing lesions was due to differences in in vivo signaling. We created a model of angiosarcoma through the sequential introduction of a temperature sensitive SV40 large T antigen and oncogenic H-ras (Arbiser et al., 1997). We demonstrated that ras can upregulate vascular endothelial growth factor (VEGF) through a phosphatidylinositol-3 kinase dependent pathway, and that blockade of PI3 kinase led to decreased tumor burden. Upon introducing a dominant negative MAP kinase kinase (MEKK) into these cells, soft agar growth was abolished, but production of matrix metalloproteinases (MMPs) was paradoxically elevated. In vivo tumor growth was also enhanced, with increased lung metastasis (LaMontagne, Jr. et al., 2000). This finding contradicted the dogma of MAP kinase activation as an oncogenic event. In order to reconcile our data with the data showing oncogenesis due to MAP kinase activation, we compared our system to the system in which MAP kinase was initially found to be oncogenic(Qiu et al., 1995; Cowley et al., 1994). MAP kinase activation is oncogenic in NIH3T3 fibroblasts, which have loss of the tumor suppressor p16ink4a. Our system, like human angiosarcoma, has defects in p53 signaling. We thus hypothesized that loss of a tumor suppressor gene dictates the signaling pathways that occur in tumors that develop consequently.

Support for a link between MAP kinase loss and p16 inactivation came from a carcinogenesis experiment, in which nickel sulfide, a well known reactive oxygen generating carcinogen, was implanted into mouse muscle. Sarcomas grow out as a result of reactive oxygen induced carcinogenesis, and in analysis of these sarcomas, we observed hypermethylation of p16ink4a and MAP kinase activation, even in tumors arising in p53 heterozygous mice (Govindarajan et al., 2002). Our conclusions from this is that reactive oxygen induced carcinogens cause hypermethylation of p16ink4a and MAP kinase activation.

While a deficiency in reactive oxygen is associated with inflammation, excessive reactive oxygen is a more common cause of inflammation (De et al., 1989). Inflammation caused by excessive neutrophil infiltration, such as psoriasis, inflammatory bowel disease, and disorders of the inflammosome are associated with elevated reactive oxygen (Hanselmann et al., 2001; Lee et al., 2011; Levine, 1992; Cassel et al., 2008). Similarly, disorders of lymphocytic inflammation can also be a source of reactive oxygen excess, and these disorders include atopic dermatitis, persistent inflammation due to hepatitis B and C, schistosomiasis, lupus, pneumonitis and forms of multiple sclerosis (Stoff et al., 2010; Lunec et al., 1994; Sanz-Cameno et al., 2006; Balasubramanian et al., 2003).

Occurrence of the reactive oxygen driven tumor in humans

Burkitts lymphoma is a high grade lymphoma that is associated with Epstein-Barr virus (EBV) in Africa and in patients with immune disorders. It is associated with a characteristic immunoglobulin c-myc translocation, and is classified as either epidemic (African) or sporadic. Of interest, virtually all sporadic Burkitt's lymphoma has mutant p53, while epidemic (EBV associated) has wild type p53 and hypermethylation of p16ink4a (Klangby et al., 1998). In our study of Burkitt's lymphoma, we found greatly elevated levels of reactive oxygen in EBV associated lymphoma compared with sporadic lymphoma (Cerimele et al., 2005). In addition, we found that MAP kinase was activated in EBV associated Burkitts lymphoma compared with sporadic Burkitts lymphoma. EBV infection induced reactive oxygen species production in lymphocytes, and reactive oxygen generation was dependent on autocrine IL-10 production, EBNA2 and LMP1 (Cerimele et al., 2005). Consistent with our findings, reactive oxygen was found to induce DNA methyltransferase 1 (DNMT1), the enzyme primarily responsible for hypermethylation of p16ink4a (Mishra et al., 2008).

Melanoma is a common solid tumor characterized by loss of p16ink4a and MAP kinase activation. In fact, germline loss of p16ink4a is the most common cause of familial melanoma (Weaver-Feldhaus et al., 1994). We found that MAP kinase is activated in 90% of all melanomas, and not in atypical nevi (Cohen et al., 2002). The most common oncogenic mutation in melanoma is Braf V600E, which activates MAP kinase, and the second most common mutation is in Nras, which also activates MAP kinase (Curtin et al., 2005). We demonstrated that MAP kinase is transforming in melanocytes, but is not sufficient to fully recapitulate the fully malignant phenotype of melanoma, implying additional pathways (Govindarajan et al., 2003). In order to elucidate additional pathways, we transfected a constitutively activated Akt into an early stage melanoma cell line, WM35. Introduction of active Akt into these cells led to a dramatic increase in reactive oxygen levels, especially superoxide, and an upregulation of VEGF. Blockade of Akt with a small molecule led to dowregulation of VEGF, rictor, an mTORC2 subunit responsible for rapamycin resistance, and SIRT1, which contributes to immortalization of tumor cells (Govindarajan et al., 2007). Most importantly, introduction of Akt led to malignant transformation in vivo. Additional groups have observed Akt amplification in melanoma, and expression of high levels of elevated Akt, especially in brain metastasis (Davies et al., 2009). One of the genes upregulated as a result of Akt expression is nox4, which other groups have described as expressed in melanoma.

The signaling pathway of hemangiomas was elucidated in part by studies of verruga peruana, an endothelial lesion induced by the bacterium Bartonella bacilliformis (Cerimele et al., 2003). Histologically, verruga peruana is nearly histologically identical to hemangiomas, and can be confuserd with hemangiomas except for the presence of intracellular bacteria. Like hemangiomas, infection of primary endothelial cells with Bartonella leads to upregulation of angiopoietin-2 (Ang-2), accompanied by upregulation of the rac signaling pathway (Verma and Ihler, 2002). Rac1 and 2 are components of NADPH oxidases, implying that hemangiomas are reactive oxygen driven tumors. We tested the functionality of nox4 in a murine model of hemangiomas. First, we established that like human hemangiomas, this model is Ang-2 dependent using an Ang trap (Perry et al., 2006a). Second, we demonstrated that siRNA to nox4 resulted in a potent inhibition of tumor growth in vivo. Third, we synthesized a novel inhibitor of nox4, fulvene 5, that had potent inhibitory activity against bend3 tumors in mice. Fulvene 5 downregulated Notch related ankyrin related protein (Nrarp), indicating that the notch signaling pathway is downstream of reactive oxygen signaling (Bhandarkar et al., 2009).

Proof of principle of reactive oxygen inhibition in humans

In order to test the efficacy of NADPH oxidase inhibition in humans, we examined FDA approved drugs that are structurally similar to diphenyleneiodonium, a well established, albeit not entirely specific NADPH oxidase inhibitor. We hypothesized that gentian violet and brilliant green would have similar activity to diphenyleneiodonium, in that these compounds have a central carbon with a cationic charge that can be delocalized (Perry et al., 2006a). Diphenyleneiodonium also has a central atom, in this case an iodine rather than a carbon. These cationic triphenylmethanes inhibit the production of reactive oxygen by both nox2 and nox4. These compounds inhibit the production of Ang-2 by bend3 hemangioma cells and cause regression of experimental hemangiomas in mice. Given these findings, we were able to assess the effects of nox inhibitors in humans.

In a trial of infants with ulcerated hemangiomas, another triphenylmethane, eosin, was applied daily under occlusion for one month. This resulted in regression of these otherwise painful lesions, thus providing a safe and effective tumor (Lapidoth et al., 2009). A second mode of treatment has also gained favor in the treatment of large infantile hemangiomas, namely propranolol, a beta adrenergic blocker. This was discovered by observing an infant who had a large hemangioma who received propranolol for treatment of cardiac disease (Leaute-Labreze et al., 2008). Surprisingly, the hemangioma of the infant regressed, leading to a clinical trial, in which propranolol was demonstrated to cause rapid regression of hemangiomas. Propranolol treatment usually requires hospitalization for infants because of the side effects of beta blockade, such as hypoglycemia, as well as monitoring of blood pressure. Interestingly, both the beta blocking active isoform of propranolol and the inactive isoform of propranolol have nox inhibitory activity, indicating that beta blockade may not be required for the activity of propranolol (Perry et al., 1992).

Growth factors induce constitutive reactive oxygen signaling

Many tumors, including hemangiomas, are thought to arise through uncontrolled proliferative responses to growth factors. In order to demonstrate this in human cells, we overexpressed platelet derived growth factor BB (PDGF-BB) in SV7tert human angiomyolipoma cells, a model of tuberous sclerosis. SV7tert cells express the receptor for PDGF-BB, namely PDGFRβ (Arbiser et al., 2002a). Overexpression of PDGF-BB resulted in upregulation of VEGF, upregulation of reactive oxygen, and in vivo transformation. Transformation was accompanied by downregulation of p16ink4a (Govindarajan et al., 2005). Overexpression of Id-1, a transcriptional suppressor, accelerated in vivo transformation, indicating that loss of p16ink4a is a necessary step to reactive oxygen mediated transformation. The high levels of reactive oxygen caused oxidative inactivation of tyrosine phosphatases, including two putative tumor suppressors, PTEN, and shp2 (Boivin et al., 2008). Reactive oxygen has also been shown to inactivate the tumor suppressor gene p53, and IkB alpha. Thus, reactive oxygen signaling leads to activation of Akt through inactivation of PTEN, activation of NFkB through inactivation of IkBalpha, and functional inactivation of p53. These are the hallmarks of the reactive oxygen driven tumor (Fried and Arbiser, 2008a). In addition, inflammatory processes can use the same processes of NFkB activation, Akt activation, and functional inactivation of p53, without malignant transformation, similar to what has been observed in hemangiomas. Thus, both malignant and inflammatory processes can be targeted by reactive oxygen inhibitors such as cationic triphenylmethanes and fulvenes.

Recognition of the reactive oxygen driven phenotype in clinical practice

In order to fully target the reactive oxygen driven phenotype, one must be able t o identify it in a paraffin section. Certain tumors, such as melanoma, glioblastoma multiforme, and pancreatic cancer have a preponderance of p16ink4a loss. However, epidemiologic data is not sufficient to make decisions in an individual patient. One marker that might have utility in this respect is cytoplasmic Wilms tumor 1 (WT1). Our initial interest in WT1 is from our discovery that WT1 is highly expressed in hemangiomas but not vascular malformations (Lawley et al., 2005). This was recently confirmed in a larger study. We also observed expression of WT1 in melanoma but not atypical nevi (Al et al., 2010; Trindade et al., 2011). Cytoplasmic WT1 has been shown to bind to actin, and may contribute to invadopodia (Dudnakova et al., 2010), and loss of WT1 has been associated with a greatly decreased ability for ras to induce tumorigenesis (Vicent et al., 2010). Thus, WT1 may both be a valuable histologic maker of reactive oxygen driven processes, as well as a pharmacologic target, as WT1, by binding actin, may regulate the F actin to G actin transition to cause tumor invasion (manuscript submitted).

Algorithm for Clinical Use

While gene arrays are highly popular, their expense currently prevents widespread use as a clinical tool. In addition, the relatively inexpensive methods of immunohistochemistry on formalin fixed sections allows spatial examination of tumor and stromal cells for the presence of markers. Therefore, we predict that the pathologist of the future will stain

tumors with a limited panel of antibodies, including p53 (to detect mutant p53), Wilms tumor 1 (WT1), to detect the ability to use reactive oxygen, and NFkB surrogates, such as ICAM1. The presence of positive WT1 and ICAM1, in the absence of p53 immunopositivity, will indicate the presence of the reactive oxygen driven tumor. P53 mutant tumors may also demonstrate NFkB activation, but not driven by reactive oxygen (Figure 1). Compounds that elevate reactive oxygen should be preferentially targeted towards tumors with mutant p53, and compounds which inhibit NADPH oxidases should be preferentially targeted towards reactive oxygen driven tumors.

Treatment of the Reactive Oxygen Driven Tumor

Two approaches have been discussed for treating tumors in terms of reactive oxygen. One method is to overwhelm the tumor with reactive oxygen, on the basis of the hypothesis that tumors that have high levels of reactive oxygen may undergo apoptosis if subjected to a reactive oxygen generating process. On the other hand, we would like to focus on the inhibition of reactive oxygen as a target, especially since we have provided proof of principle in human disease. As mentioned earlier, reactive oxygen is capable of reversibly inactivating proteins involved in NFkB signaling. This means that by pharmacologic means, one can downregulate NFkB by downregulating the production of superoxide. This is of major pharmacologic importance, because one can thus achieve selective inhibition of cells that rely on superoxide for NFkB mediated survival, and in the case of tumors, NFkB mediated immune evasion.

Major mediators of both inflammation and tumor survival include TGF beta, TNF alpha, and CD40, all of which when activated in the tumor milieu, result in reactive oxygen generation and NFkB activation. Mechanisms include oxidative inactivation of IkB, resulting in nuclear translocation of p50/p65 subunits of NFkB (canonical activation) (Schreck et al., 1991). Canonical activation of NFkB in a constitutive manner has been observed in melanoma, glioblastoma, hematologic malignancies, as well as many other solid tumors (Torisu-Itakura et al., 2007; Yamini et al., 2007). Inducible canonical activation of NFkB has been observed in inflammatory processes, such as arthritis, inflammatory bowel disease, multiple sclerosis, scleroderma, atopic dermatitis and psoriasis, among other disorders. We have shown the efficacy of gentian violet, a topical NADPH oxidase inhibitor, in atopic dermatitis, an extremely common disorder in which bacterial toll receptor activation combines with TNF alpha to perpetuate inflammation (Stoff et al., 2010). Not all inflammatory processes are reactive oxygen mediated, a case in point is chronic granulomatous disease (CGD), in which inflammatory granulomata are a major cause of inflammation. CGD results from loss of subunits associated with nox2, thus it is a reactive oxygen deficient disorder. Biomarkers are needed in order to determine which inflammatory processes would respond best to NADPH oxidase inhibition. Based upon the currently available data, the major NADPH oxidases that merit targeting are nox1, which generates primarily superoxide, and nox4, which primarily generates hydrogen peroxide.

NOX 1 and 4

Briefly, seven proteins and several activator/organizer proteins comprise to make up the NADPH oxidase or NOX family: NOX1–5, DUOX1, DUOX2, p22phox, p47phox, NOXO1/ p67phox, and NOXA1/p40phox. NOX proteins (NOX1–5, DUOX1, DUOX2) are transmembrane proteins that carry across electrons, reducing oxygen in to superoxide or ROS. NOX proteins (1–4) with the exception of NOX5 require p22Phox in order to produce ROS. NOX1–3 also depend on cytosolic subunits NOXO1 (NOX organizer 1 _ p47phox homolog (NOXO2)) and NOXA1(NOX activator 1 _ p67phox homolog) for activation (Bedard and Krause, 2007) (Lambeth et al., 2007). We are focusing on nox1 and nox4 since these two enzymes are currently the most implicated in cancer and pathologic inflammation (Figure 2).

NOX1 is located on chromosome Xq22 and is well documented as highly expressed in colon epithelium and in colon cancer cell line CaCO-2. Recent work by Shinohara et al draws a complex relationship between NOX1 and the oncogene Ras. The Shinohara group describes Ras activated NOX1 as a mediator of cellular invasion, tumorogenesis and angiogenesis. Experimental results drawn from K-ras transformed normal rat kidney cells (KNRK) and colon cancer CaCO-2 attribute NOX1 activation through a Ras/ERK/MEK/GATA-6 pathway. The subsequent upregulation of NOX1/ROS induces an NFKB mediated cellular invasion through an increase production of metalloprotease-9 (MMP-9), where upregulation of cytosolic subunits NOXO1 and NOXA1 play a significant role in NOX1/ROS mediated MMP-9 over-expression. MMPs degrade extracellular matrix proteins allowing for increase invasive properties. NFKB is also highly activated in adenocarcinoma cells expressing NOX1. NOX1/ROS mediates Ras-induced NFKB signaling by increasing IkBalpha phosphorylation by IKKalpha and degrading IkBalpha resulting in NFKB activation, a process reversed by NOX1 siRNA. NOX1 also mediates the downregulation of Rho via EGF and increase cellular mobility. Activation of p190RhoGAP by EGF is attributed to the downregulation or Rho activity, a process also reversed by NOX1 siRNA. This is consistent with the opposing roles of rac isoforms and rhoA, with rhoA activation being associated with low levels of reactive oxygen, and rac activation being associated with elevated levels of reactive oxygen (Shinohara et al., 2007). Nox1 may also be the major nox isoform involved in tumor angiogenesis. Nox1 deficiency, but not nox4 deficiency, impaired angiogenesis through a PPARα dependent pathway (Garrido-Urbani et al., 2011).

NOX4 is located on chromosome 11q14.2-q21 and has been well noted to be highly expressed in kidneys, blood vessels as well as attributed to malignant melanoma and pancreatic carcinoma. Like NOX1–3, NOX4 is p22phox dependent for the generation of ROS. However, NOX4 does not require cytosolic subunits as in the case for NOX proteins 1–3. NOX4 in melanoma has been closely linked to the cell survival via the activation of AKT and NFKB via ROS, as well as, the subsequently induced hypoxic factor HIF2 alpha leading to upregulation in angiogenesis via VEGF, ANG2 (Fried and Arbiser, 2008b). Blockade of NOX4 has been associated with decreased growth of several neoplasms.

Mochizuki et al, indicate NOX4 in pancreatic adenocarcinoma as mediating survival via ROS/AKT/ASK1 pathway. Phosphorylation of apoptosis signal-regulating kinase 1(ASK1)

via NOX4 induced AKT gives addition insight into how NOX4/AKT may act to inhibit apoptosis and increase cell survival. Through the pancreatic cancer cell line PANC-1, Mochizuki group reveals that inhibition of AKT phosphorylation of ASK1 alone was sufficient in inducing apoptosis (Mochizuki et al., 2006). NOX4 has also been implicated in melanoma growth from two sources. First, we have demonstrated that transfection of Akt results in induction of NOX4 in early melanoma cells. Second, introduction of NOX4 siRNA leads to an inhibition of melanoma growth in vivo in two separate studies (Brar et al., 2002; Yamaura et al., 2009). Nox4 has been implicated in renal cell carcinoma as a direct stimulator of HIF2a, which is of physiologic relevance, as tumors that express high levels of HIF2a, but not HIF1a, have a worsened prognosis (Maranchie and Zhan, 2005; Block et al., 2007; Gordan et al., 2008). We have found an essential role for NOX4 in hemangioma growth, and inhibitors of NOX4 result in hemangioma growth inhibition, including both fulvenes and triphenylmethanes (Bhandarkar et al., 2009; Garrido-Urbani et al., 2011; Perry et al., 2006b). Intriguingly, a recent report has shown that NOX4 mediates cell death in head and neck squamous cell carcinoma, upon treatment with EGFR inhibitors (Orcutt et al., 2011). All of the cell lines used in this study have mutant p53, highlighting the role of mutant p53 versus p16 loss in the choice of antioxidant versus prooxidant signaling pathways.

Future trends

The identification of the reactive oxygen driven phenotype, both in inflammation and tumorigenesis, has clinical consequences. Pathologic conditions that are characterized by excessive reactive oxygen will likely be responsive to reactive oxygen inhibitors, while conditions with defective reactive oxygen may be responsive to reactive oxygen inducers. The epidemiology of reactive oxygen driven inflammation and tumorigenesis is well established. We have found histologic tools that can be used on paraffin in humans sections to determine the presence of the reactive oxygen driven phenotype. Finally, we and others have developed small molecule inhibitors of nox enzymes that are active in animal models and in human patients. Wider applications of these principles may lead to improved therapies for inflammation and cancer.

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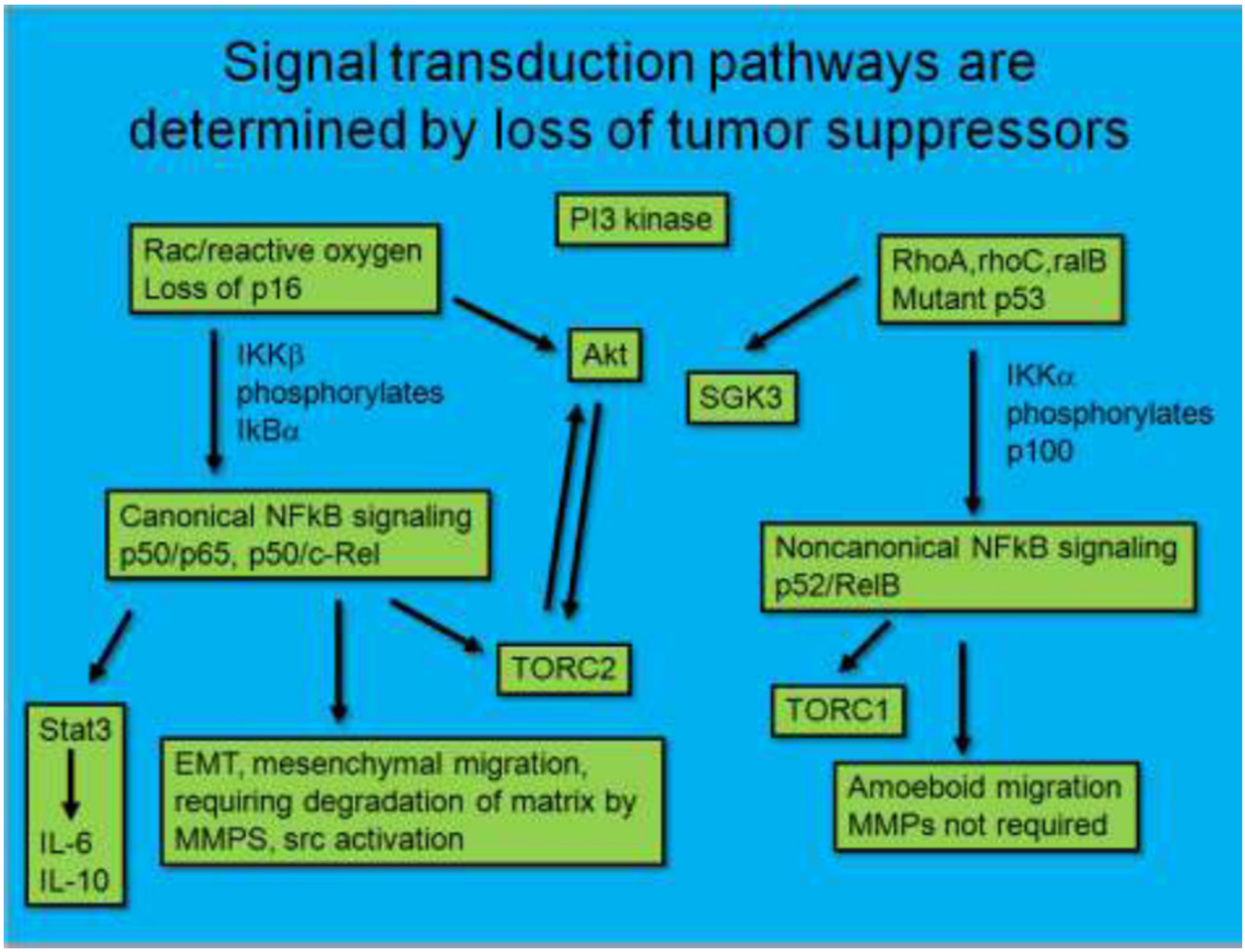

Figure 1.

Dependence on signaling pathways based upon tumor suppressor status. Tumors that are deficient in p16ink4a are more likely to use a reactive oxygen/rac/NFkB signaling pathway, while tumors that have mutant p53 are more likely to use a noncanonical NFkB signaling pathway and phosphoinositol-3 kinase dependent but Akt independent pathway.

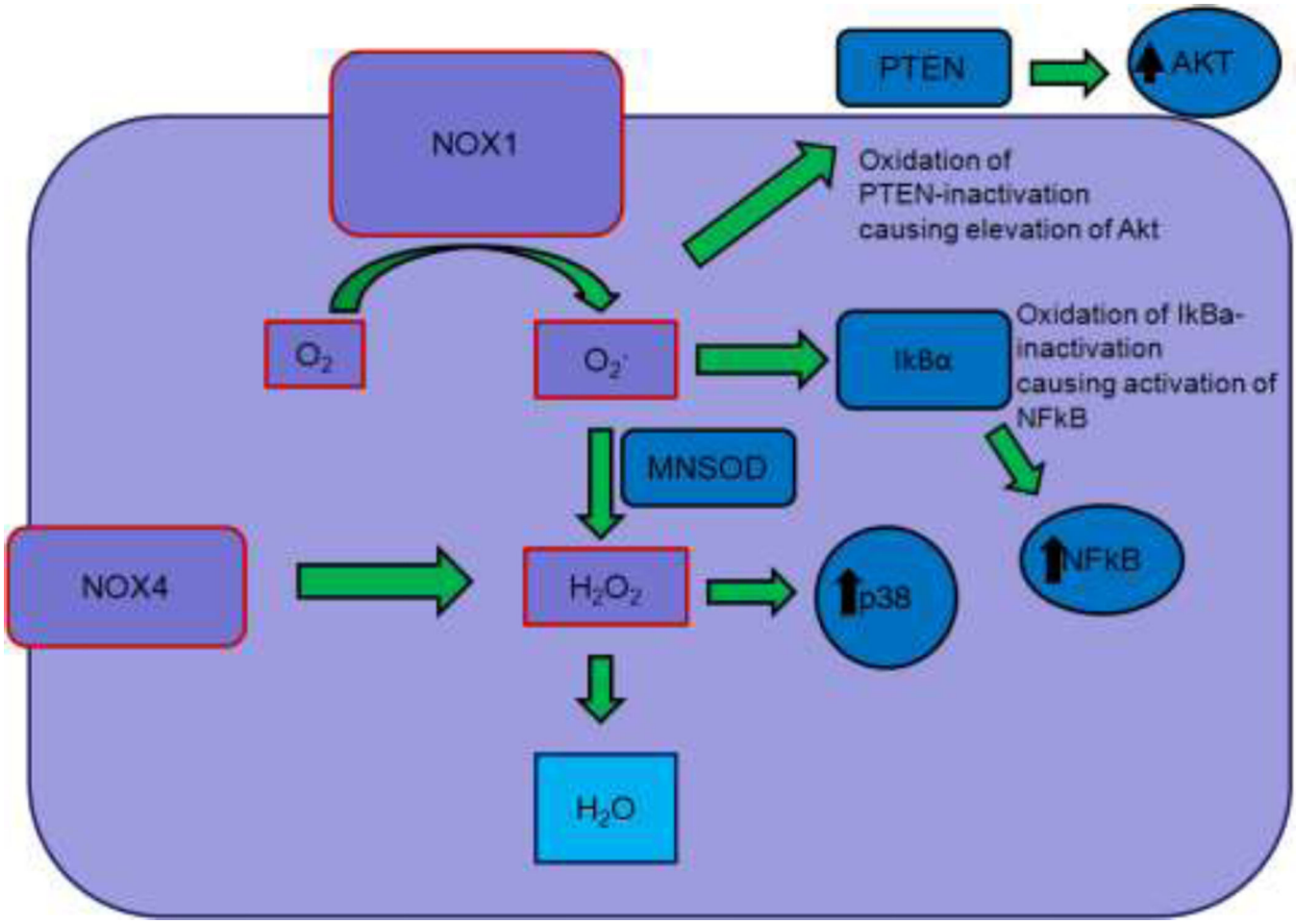

Figure 2.

Diagram of reactive oxygen induced signaling, highlighting the role of Nox1 derived superoxide and nox4 derived hydrogen peroxide in activating signaling pathways, most notably Akt and NFkB.