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Oxidative Stress and Redox-Signaling in Renal Cell Cancer

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1. Introduction

Worldwide, approximately 150,000 people are diagnosed with Renal Cell Carcinoma (RCC) and 78,000 deaths are reported each year with the incidence on the rise (Jemal et al, 2010). Renal tumors are classified according to the "Heidelberg classification" where the tumors are separated based on their location within the nephron and linked to morphologic and genetic abnormalities (Schullerus et al, 1997). While most cases of RCC occur sporadically, inherited predisposition to renal cancer accounts for ~5% of cases. Hereditary and sporadic gene mutations associated with renal carcinoma include, von Hippel-Lindau (*VHL*) (Maher & Kaelin, 1997; Tory et al, 1989; Latif et al, 1993), tuberous sclerosis 2 (*TSC2*), (Washecka & Hanna, 1991), fumarate hydratase (*FH*) (Pfaffenroth & Linehan, 2008), succinate dehydrogenase (*SDH*) (Vanharanta et al, 2004; Henderson et al, 2009; Ricketts et al, 2008), MET (Schmidt et al, 1997; Lubensky et al, 1999), and Birt-Hogg-Dube' (*BHD*) (Pavlovich et al, 2002; Khoo et al, 2001, Schmidt et al, 2001). The diverse nature of these genes and the histologically distinct tumors they give rise to implicates various mechanisms and biological pathways in renal tumorigenesis. On the cellular level, inactivation of common pathogenic pathways and mechanisms involve oxidative stress. Oxidative stress is caused by an imbalance between the production of reactive oxygen species and the cells ability to neutralize the reactive intermediates. Adverse effects occur when the excess reactive oxygen species damage a cell's lipids, protein or DNA; together contributing to genomic instability and tumorigenesis. Additionally, reactive oxygen species can serve as important upstream regulators as well as downstream mediators of action through redox-signaling. Two major sources of oxidative stress in the kidney include the Mitochondria and NAD(P)H oxidases of the Nox family. Unlike natural byproducts of mitochondrial metabolism or mitochondrial dysfunction, reactive oxygen species generated by Nox oxidases function as signaling molecules that initiate and/or modulate different regulatory pathways involved in tumorigenesis and metastasis. Clinically, efforts to target specific enzymatic sources of reactive oxygen species production, that result in alterations of signaling and metabolism, represents novel therapeutic approaches to treat renal cancer. This chapter will review the links between genes inactivated in RCC that lead to enhanced oxidative stress, mediated by different enzymatic sources, and the biological pathways activated by redox-sensitive signaling molecules involved in cell growth, cell survival, and metastasis in RCC.

2. Sources of oxidative stress in renal cancer

Renal cell carcinoma, as is the case in many cancers, demonstrate oxidative stress (Szatrowski et al, 1991). Oxidative stress is defined as an imbalance between the production of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates (Fridovich, 1978). Oxidative stress not only causes direct and irreversible oxidative damage to macromolecules but also disrupts key redox-dependent signaling processes. Reactive oxygen species include hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), peroxynitrite ($ONOO^-$), and superoxide ($O_2^{\cdot-}$), many of which have been detected in renal cell carcinoma (Wickramasinghe, 1975; Block et al, 2007, 2010). Intracellular generation of the superoxide anion $O_2^{\cdot-}$ occurs, in part, by the semi-ubiquinone compound of the mitochondrial electron transport chain (Cadenas & Davies, 2000; Evans & Halliwell, 1999) and through NADPH-oxidases of the Nox family (Nox) (Babior, 1999; Vignais, 2002). Superoxide can interact with nitric oxide (NO) to produce peroxynitrite ($OONO^-$), a very reactive intermediate. Superoxide is converted into hydrogen peroxide enzymatically by the cytosolic antioxidant, superoxide-dismutase-1 (SOD1) or the mitochondrial superoxide-dismutase-2 (SOD2) and is then converted to water by glutathione peroxidase (GPX); however, this conversion is not 100% efficient and expression and activity of SOD1 is reduced in conventional renal cell carcinoma (Sarto et al, 1999; Fukai & Ushio-Fukai, 2011). Superoxide poorly crosses biological membranes (Evans & Halliwell, 1999); however, hydrogen peroxide can easily diffuse across biological membranes and is then removed by the antioxidant, catalase. Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) can react to form a highly reactive and damaging hydroxyl ($\cdot OH$) radical, which can not diffuse from the site of generation and quickly damages surrounding macromolecules such as amino acids, carbohydrates, lipids, and nucleic acids. Oxidative damage on nucleic acids form adducts such as deoxyguanine (8-OH-dG), which if not cleared can potentially generate mutations (Novo & Parola, 2008). 8-OH-dG is often used as an intracellular marker of oxidative stress. Together, overproduction of reactive oxygen species and/or alterations of the antioxidant system are key pathological triggers of cancer. Major sources of reactive oxygen species in renal cell carcinoma are NADPH oxidases of the Nox family and mitochondria. Unlike the mitochondria, which generate reactive oxygen species as a byproduct of cellular metabolism, NADPH oxidases of the Nox family generate reactive oxygen species that modulate redox-sensitive cellular responses and are essential mediators of normal cell physiology. However, as discussed below, excessive reactive oxygen species production by an overactive NADPH oxidase system, likely mediates constitutive activation of signaling pathways involved in the initiation and progression of renal carcinogenesis. This occurs through the selective oxidation of specific signaling enzymes/proteins that are linked to processes such as activation of transcription factors, secretion of cytokines or altering signaling proteins such as protein kinases and phosphatases. Redox research is providing evidence that increased and/or sustained levels of oxidative stress play a large role in the genesis of human cancers, including renal cancer.

2.1 NAD(P)H Oxidases of the Nox family as a source of oxidative stress in renal cell carcinoma

Figure 1. NAD(P)H oxidases of the Nox family are major sources of reactive oxygen species in renal cancer. Nox oxidases have six N-terminal transmembrane regions which contain four heme-binding histidines and in the C-terminal cytosolic region, they have an FAD and

a NADPH-binding domain which together catalyse the reduction of molecular oxygen, using NADPH as an electron donor, to generate superoxide, (O_2^-) which is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Although these oxidases are proposed to play a role in a variety of signaling events, such as cell growth, cell survival, oxygen sensing and inflammatory processes, their *bona fide* functions and regulation, as well as molecular composition, are largely unknown. Early studies on NAD(P)H oxidases were performed in neutrophils and phagocytic cells, investigating the respiratory burst NAD(P)H oxidase system. The molecular composition of the phagocyte respiratory burst oxidase or phagocyte NAD(P)H oxidase consists of two plasma membrane-associated proteins, gp91phox (the catalytic Nox subunit, now called Nox2) and the small regulatory subunit, p22phox, which comprise flavocytochrome b558. In addition to the membrane bound components, cytosolic factors, p47phox p67phox p40phox, and the small GTPase Rac are also necessary to activate the phagocyte NAD(P)H oxidase. Upon activation, the gp91phox phagocyte Nox oxidase generates a “burst” of reactive oxygen species, which functions in immunity. Homologs of Nox2, termed Nox (for NAD(P)H oxidase) proteins have been identified in somatic cells and generate reactive oxygen species at a much lower concentration than the phagocyte Nox oxidase. To date, the Nox family comprises seven members: Nox1-5 and the dual oxidases Duox-1 and -2 (Suh et al, 1999; Royer-Pokora et al, 1986; Cheng et al, 2001; Geiszt et al, 2000; Banfi et al, 2001; Dupuy et al, 1999; Deken et al, 2000). For the purpose of this chapter, Nox isoforms will only be considered. Nox1, Nox2, and Nox4, are the NAD(P)H oxidase isoforms that are predominantly expressed in the various renal cells (Bondi et al, 2010; Gorin et al, 2003, 2005; Block et al, 2007, 2009; Eid et al, 2009). The isoform Nox4/Renox was cloned from the kidney (Geiszt et al, 2000; Shiose et al, 2001). It is a 578-amino-acid protein that exhibits 39% identity to the phagocyte Nox2 with special conservation in the six membrane-spanning regions and binding sites for NAD(P)H, flavin adenine dinucleotide (FAD), and heme, the electron transfer centers that are required to pass electrons from NAD(P)H to oxygen to form superoxide and hydrogen peroxide (Lassegue & Griendling, 2010; Bedard & Krause, 2007; Brown & Griendling 2009; Geiszt, 2006; Selemidis et al, 2008; Geiszt et al, 2000; Shiose et al, 2001). The dehydrogenase domain of Nox4 exists in a conformation that allows spontaneous transfer of electrons from NAD(P)H to FAD, suggesting the enzyme has constitutive activity that is regulated primarily at the level of its expression in response to various stimuli (Nisimoto et al, 2010). Additional evidence suggests that in the presence of certain stimuli, Nox4 activity is enhanced when bound to p22phox, but does not require cytosolic subunits that are essential to activate other Nox isoforms (Bedard et al, 2007; Geiszt, 2006; Selemidis et al, 2008; Ambasta et al, 2004; Martyn et al, 2006). The localization of Nox4 may be cell type specific and has been documented to localize to intracellular membranes of the endoplasmic reticulum, focal adhesions and nucleus (Lassegue & Griendling, 2010; Bedard et al, 2007; Brown et al, 2009; Martyn et al, 2006; Pedruzzi et al, 2004; Hilenski et al, 2004). Nox4 harbors internal sequences that are predictive of a mitochondrial targeting sequence. Indeed, in the kidney, Nox4, unlike other Nox isoforms Nox4 localizes to the mitochondria (Block et al, 2009; Kuroda et al, 2010). This finding may suggest novel cross talk of the Nox oxidases and mitochondria in renal cancer. Nox1 is expressed in renal proximal tubular cells, glomerular mesangial cells, and podocytes. Activation mechanisms for Nox1 are similar to those of Nox2 and involve complex formation with regulatory cytosolic subunits upon agonist stimulation. However, in contrast to Nox2, Nox1 primarily interacts with the p47phox homolog, NoxO1 (Nox organizer 1), the p67phox homolog, NoxA1 (Nox activator 1), and

Rac upon activation (Lassegue & Griendling, 2010; Lambeth, 2007; Bedard & Krause, 2007; Brandes & Schroder, 2008; Geiszt, 2006; Selemidis et al, 2008). The expression of Nox regulatory subunits, p22^{phox}, p47^{phox} and p67^{phox} are also expressed in renal cells (Jones et al, 1995). While Nox4 and p22^{phox} over-expression seems to be a feature of renal cancer cells, ongoing studies are addressing the mechanisms by which Nox enzymes play a causal role in the renal cancer phenotype. Nox-dependent effects on cell division, angiogenesis, cell survival, mitogen, and cytokine signaling in a subset of human cancers provide putative mechanisms by which Nox enzymes may be linked to cancer development. For example, Nox1 over-expression transforms normal fibroblasts and creates a cell that is tumorigenic in athymic mice (Suh et al, 1999). Furthermore, Nox1 triggers an angiogenic switch and converts tumors from dormant to aggressive growth (Arbiser et al, 2002). Nox4 was found to regulate growth of malignant melanoma cells and to inhibit apoptosis of pancreatic cancer cells (Mochizuki et al, 2006; Vaquero et al, 2004). Nox5 mediates growth of prostate cancer cells (Brar et al, 2003). Overexpression of p22^{phox} in normal proximal tubular epithelial cells can activate signaling pathways known to be constitutively active in the majority of renal cancers (Block et al, 2010). Nox activity is higher in renal cell carcinoma

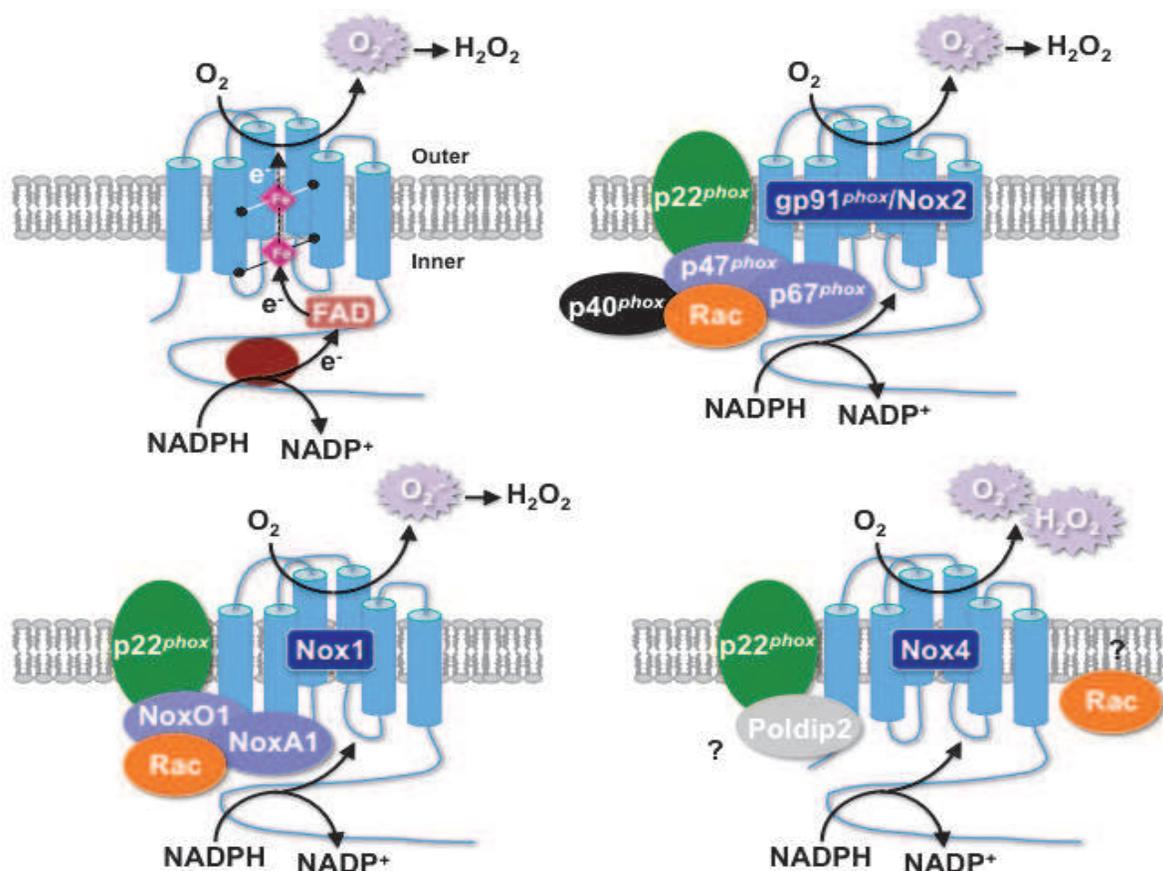


Fig. 1. Structure and molecular organization of the nicotinamide adenine dinucleotide phosphate, NAD(P)H oxidases of the Nox family. The top left panel illustrates the topology and the enzymatic reaction catalyzed by the Nox enzymes. The other panels represent the molecular structure of the different isoforms of Nox oxidases predominantly expressed in renal carcinoma cells, gp91^{phox}/Nox2, Nox1, and Nox4. All Nox proteins can form a complex with p22^{phox}, but the cytosolic subunits differ from the Nox oxidase isoforms. FAD, flavin adenine dinucleotide; H₂O₂, hydrogen peroxide; O₂^{•-}, superoxide.

compared to normal proximal tubular epithelial cells and the expression of cytosolic SOD1 is reduced (Block et al, 2010). It has been demonstrated that superoxide is the main reactive oxygen species necessary for maintaining the expression of a critical protein involved in renal carcinogenesis, HIF-2alpha (Block et al, 2010).

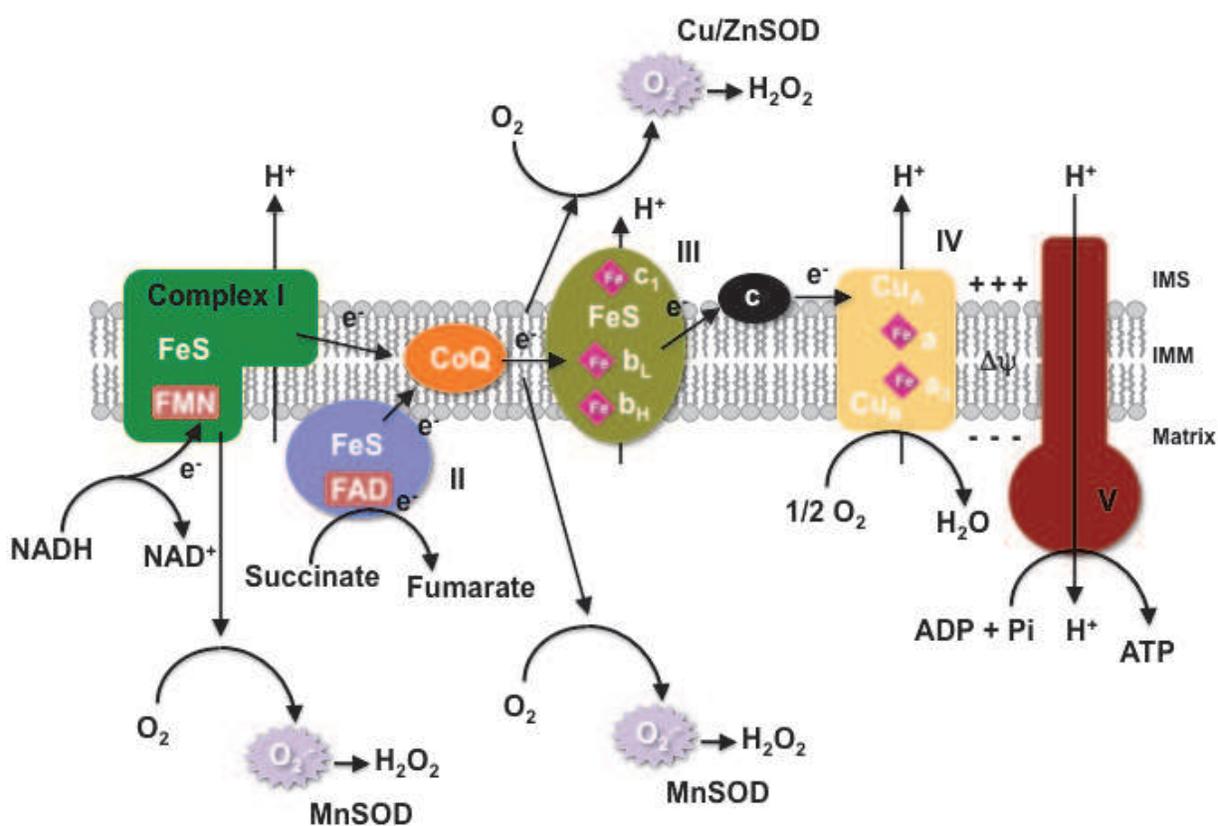


Fig. 2. Production of ROS by the mitochondrial electron-transport chain. IMS, intermembrane space; IMM, inner mitochondrial membrane; $\Delta\psi_m$, mitochondrial membrane potential.

2.2 Mitochondria as a source of oxidative stress in renal cell carcinoma

Mitochondria play a central role in the generation of reactive oxygen species in cells and tissues. Aerobic energy metabolism relies on oxidative phosphorylation, a crucial process by which the oxidoreduction energy of mitochondrial electron transport is converted to the high-energy phosphate bond of ATP. During mitochondrial oxidative phosphorylation, superoxide anion and hydrogen peroxide can be formed. In normal respiratory cells, approximately 5% of electrons flowing through the electron transport chain can be diverted to form O₂^{•-} at the levels of complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome c oxidoreductase) (Cadenas & Davies, 2000; Halliwell et al, 1999) (Figure 2). O₂^{•-} is then converted by mitochondrial SOD (SOD2) into hydrogen peroxide (H₂O₂). Mitochondrial dysfunction, enhanced metabolism, or genetic alterations in mitochondrial DNA are potential mechanisms by which mitochondria-dependent reactive oxygen species generation is enhanced in cancer cells. Within the mitochondria, elements that are particularly vulnerable to free radicals include lipids, proteins, and mitochondrial DNA (mtDNA). Mitochondrial DNA is highly susceptible to damage because it is not

protected by histones and is directly exposed to reactive oxygen species generated by the respiratory chain and DNA repair capacity is less efficient in the mitochondria. Free radical damage to mitochondrial proteins decrease their affinity for substrates or coenzymes resulting in reduced function and thus the production of more free radicals, which cause additional mitochondrial damage. Mitochondrial dysfunction is determined by a decrease in mitochondrial membrane potential and reduction of mitochondrial respiration with decreased ETC complex I and III activity while increasing mitochondrial-produced hydrogen peroxide. Tumor cells shown to exhibit mitochondrial dysfunction are those that have mutations in the tricarboxylic acid (TCA) cycle enzymes succinate dehydrogenase (SDH) or fumarate hydratase (FH). Electron microscopy of renal tumors has also demonstrated changes in the number, shape and function of mitochondria (Tickoo et al, 2000). Mitochondrial dysfunction in renal oncocytomas (BHD) are linked to mutations in subunits of complex I (Mayr et al, 2008). Additionally, chromophobe renal carcinoma exhibit abnormal mitochondria with altered cristae suggesting compromised mitochondrial function (Moreno et al, 2005). Alternatively, the production of reactive oxygen species may be altered by changes in mitochondrial metabolism. Cancer cells have enhanced expression of glucose transporters allowing increased consumption of glucose with high detectable levels of secreted lactate. This phenomenon, known as the “Warburg” effect, occurs when glucose is processed to pyruvate (via glycolysis) and pyruvate is converted into lactate in lieu of acetyl CoA (the primary intermediate of citric acid cycle) giving rise to glycolytic ATP production in the presence of oxygen (Warburg et al, 1924; Bui & Thompson, 2006; Brahim-Horn et al, 2007). Overall, this altered metabolism, known as “tumor metabolism”, mediates mitochondrial dysfunction and enhanced mitochondrial-dependent reactive oxygen species generation leading to enhanced cell growth and cell survival.

3. Gene inactivation and cellular factors that give rise to oxidative stress in RCC

Gene inactivation associated with oxidative stress

3.1 Loss of VHL

The von Hippel-Lindau gene (*VHL*) is inactivated in ~80% of renal cell carcinomas due to inherited or sporadic point mutations, deletions or promoter hypermethylation (Gnarra et al, 1994; Pfaffenroth & Linehan, 2008). Histologically, VHL-deficient tumors present as clear cell as the cytoplasm of these tumors are rich in lipids and glycogen, which provide the characteristic clear cytoplasm. Clear cell renal carcinoma is histologically the most common form of renal cancer and is likely derived from the renal tubular epithelium. The importance of VHL inactivation in renal carcinogenesis is underscored by the finding that restoration of VHL function in VHL-defective renal carcinoma cells suppresses tumor formation in nude mice (Gnarra et al, 1996; Iliopoulos et al, 1995). VHL is the substrate recognition module of an E3 ubiquitin ligase complex that contains elongin B, elongin C, Cul2, and Rbx1 (Kibel et al, 1995; Kamura et al, 1999). This complex targets the alpha subunits of the heterodimeric transcription factor HIF (hypoxia-inducible factor) for polyubiquitination and proteasomal degradation. Cells lacking wild-type VHL fail to degrade HIF-alpha subunits, thus hypoxia-inducible gene products are constitutively overproduced. Loss of VHL, and clear cell renal carcinoma in general, are associated with enhanced oxidative stress, mediated in large part by Nox oxidases. Nox-dependent superoxide generation is higher in cultured VHL-deficient

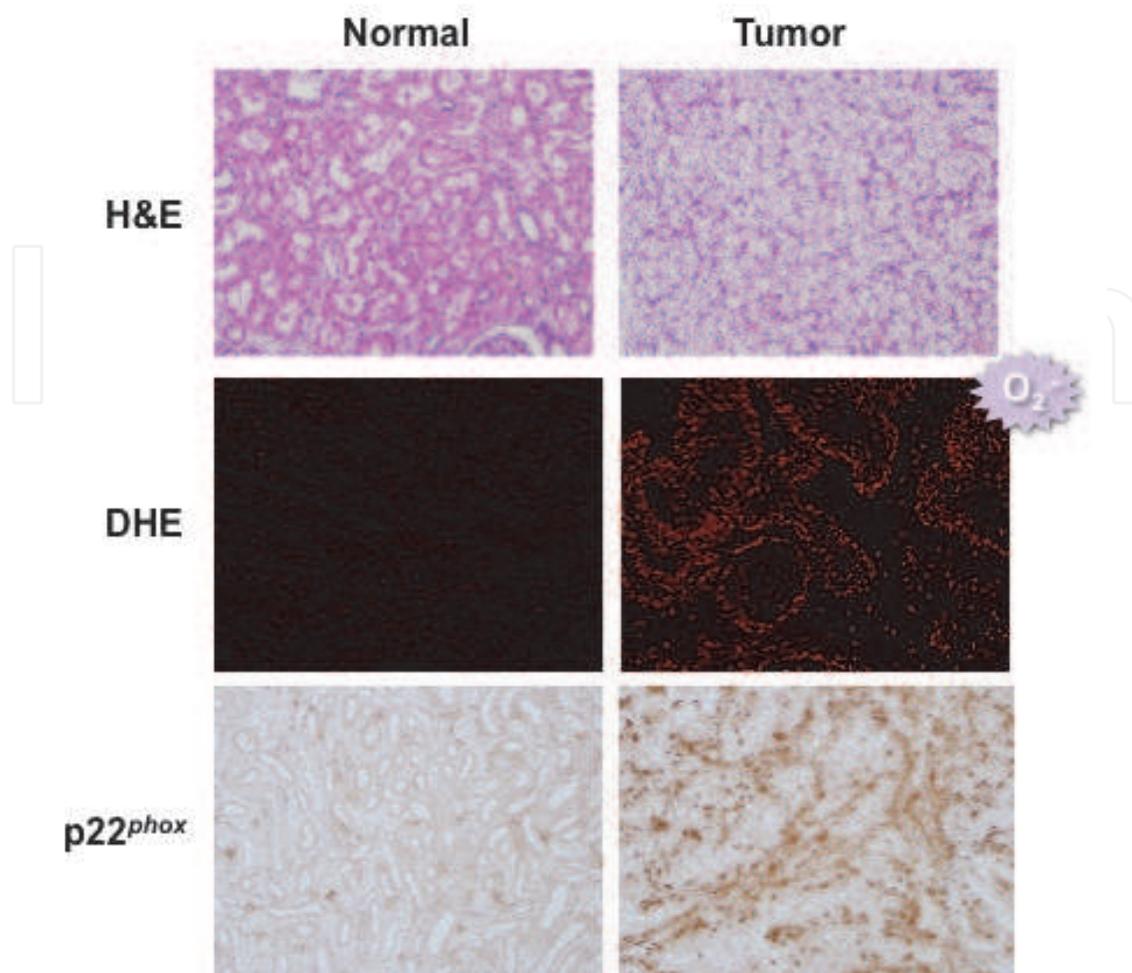


Fig. 3. p22^{phox} protein expression and superoxide production is elevated in RCC tumors compared to normal adjacent renal tissue. Adapted from Block et al, 2010. *Top panel*, H&E staining. *Middle panel*, Detection of superoxide (O₂⁻) in frozen 30-um-thick RCC sections, with dihydroethidium (DHE). *Bottom panel*, p22phox was detected by immunoperoxidase staining.

RCC cells compared to normal epithelial cells, mediated through p22phox-based Nox oxidases, **Figure 3**, (Block et al, 2007, 2010). p22phox protein expression, the Nox regulatory subunit necessary for Nox4 and Nox1 activation, is higher in VHL-deficient cultured renal cancer cells and in human renal tumors compared to normal controls (Block et al, 2007, 2010). Although the mechanism has not been fully defined, p22phox is an ubiquitinated protein and can associate with the von Hippel-Lindau protein *in vivo*, suggesting that p22phox-based Nox oxidase complexes may be stabilized upon the loss of the tumor suppressor protein, von Hippel-Lindau (pVHL). The Nox catalytic subunit, Nox4 is also overexpressed in VHL-deficient cells and in a subset of human RCCs at the mRNA and protein level (Maranchie & Zhan, 2005; Block et al, 2007, 2010). Although the mechanisms remain unclear, the Nox4 promoter harbors hypoxia responsive elements (HRE) known to be transcriptionally activated by HIFs (Diebold et al, 2010). Nox1 is expressed in renal tubular epithelial cells and is overexpressed in a subset of human RCC tumors compared to normal adjacent tissue (Block, 2010). Nox1 play a role in Nox-dependent reactive oxygen species and the genesis of RCC. Finally, it is clear in other cell types that Nox subunits and

Nox-derived ROS can be upregulated/activated by growth factors (Gorin et al, 2005; Bondi et al, 2010; Meng et al, 2008; Michaeloudes et al, 2011; Sturrock et al, 2006). Although the role of growth factor-induced Nox expression has not been explored, it is likely an alternative mechanism for enhanced Nox-derived reactive oxygen species in RCC.

3.2 TSC2

Tuberous sclerosis complex (TSC) is a multi-system genetic disease that causes tumors to form in several different organs, primarily in the kidney, brain, eyes, heart, skin and lungs. Tuberous sclerosis complex, like von Hippel-Lindau disease, are autosomal dominant tumor suppressor syndromes that can exhibit similar renal phenotypes and seem to share some signaling pathway components. TSC is caused by mutations in either the *TSC1* gene, located on chromosome 9 (Slegtenhorst et al, 1997) or the *TSC2* gene, located on chromosome 16 (European Chromosome 16 Tuberous Sclerosis Consortium, 1993). The TSC complex integrates cellular signaling inputs such as growth factors and cellular energy supply and regulates cell growth, proliferation, and survival. *TSC1* encodes hamartin and *TSC2* encodes tuberin, which form a heterodimer that inhibit mammalian target of rapamycin (mTOR) activity. mTOR is a key upstream regulator of protein synthesis activated in the majority of renal cancers and is discussed in detail below. Mutations in *TSC1* or *TSC2* genes give rise to tumors exhibiting increased phosphorylation of mTOR substrates and readouts of active mRNA translation, p70S6 kinase and 4E-BP1. Inactivation of *TSC1/2* results in HIF accumulation through increased HIF mRNA translation by activated mTOR signaling. Rodent models harboring heterozygous mutations in the *TSC2* gene develop spontaneous RCC, due to loss of heterozygosity (LOH). Kidneys of *TSC2*^{-/-} rats demonstrate higher levels of the oxidative stress marker, 8-oxo-dG. In humans, between 60 and 80% of TSC patients have benign renal tumors called angiomyolipomas (AML) (Crino et al, 2006). These tumors are composed of vascular tissue (angio-), smooth muscle (-myo-), and fat (-lipoma). The discrepancy of benign and malignant *TSC2*-deficient tumors in the human and rodent disease respectively is unclear. In human AMLs, upregulation of the tumor suppressor phosphatase and tensin homolog (PTEN) by HIF-1 alpha was demonstrated to reduce Akt activation suggesting that PTEN may safeguard against developing malignant tumors in patients with TSC deficiency (Mahimainathan et al, 2009). A minority of TSC patients progress to renal cell carcinoma. Although the mechanisms remain unclear, oxidative stress may play a role. The DNA lesion caused by oxidative stress, 8-oxoguanine (8-oxy-dG), is normally excised and repaired by 8-oxoguanine DNA glycosylase 1 (hOGG1), which localizes in the nucleus and the mitochondria. Down regulation of OGG1 has also been linked to TSC-deficiency (Habib et al, 2008, 2009). Alternatively, *OGG1* is located on a chromosome region often demonstrating LOH, 3p25-26 in renal cell carcinoma (Gokden et al, 2008). Although genetic mutations in *TSC2* have not been detected in conventional clear cell renal carcinoma, Nox-dependent reactive oxygen species generation has been identified to post-translationally inactivate tuberin (Block et al, 2010). Taken together, reactive oxygen species may play a role in TSC inactivation, downregulation of OGG and DNA and lipid damage.

3.3 Tricarboxylic acid (Krebs) cycle genes, fumarate hydratase (FH)/succinate dehydrogenase (SDH)

The tricarboxylic acid (TCA)/Krebs cycle is part of a metabolic pathway coupled to mitochondrial oxidative phosphorylation that converts nutrients to energy in aerobic cells.

The fumarate hydratase (*FH*) and succinate dehydrogenase (*SDH*) genes encode mitochondrial TCA cycle enzymes that play an essential role in energy production by catalyzing the conversion of fumarate to malate and succinate to fumarate respectively. Individuals who harbor germline mutations in either of these TCA cycle enzymes have an increased risk for developing renal tumors. Mutations in fumarate hydratase (*FH*) gene give rise to a rare form of hereditary leiomyomatosis and renal cell carcinoma (HLRCC). Renal tumors arising from genetic loss of *FH* range from type 2 papillary to tubulo-papillary to collecting-duct carcinomas. These tumors have significantly impaired oxidative phosphorylation and thus demonstrate aerobic glycolysis (Warburg effect) and are aggressive (Warburg et al, 1924). Positron emission tomography (PET) imaging demonstrates high glucose uptake in *FH*-deficient renal tumors lead to enhanced reactive oxygen species, mediated by a p47phox-based Nox oxidase, suggesting a role for the Nox oxidase isoform, Nox1 or Nox2 (Sudarshan, 2009). There is no evidence of genetic mutations in *FH* in sporadic conventional renal cell carcinoma; however, it has been demonstrated that mRNA and protein levels of *FH* are reduced in clear cell renal carcinoma (Sudarshan et al, 2011). Reduced levels of fumarate hydratase in clear cell renal carcinoma is associated with stabilized HIF-2 α levels, likely mediated through an Akt-dependent mRNA translational pathway (Sudarshan et al, 2011). Additionally, overexpression of *FH* in VHL-deficient cells reduced cell invasion, suggesting that reduced levels of *FH* play a role in metastasis in clear cell renal carcinoma. Succinate dehydrogenase, *SDH* (complex II) is a functional member of both the Krebs cycle and the aerobic respiratory chain. Complex II couples the oxidation of succinate to fumarate in the mitochondrial matrix with the reduction of ubiquinone in the membrane (Cecchini et al, 2002). Mutations of the nuclear encoded genes of the mitochondrial oxidative phosphorylation complex, succinate dehydrogenase B gene (*SDHB*) are associated with renal cell carcinoma (Vanharanta et al, 2004; Henderson et al, 2009; Ricketts et al, 2008). There is no detectable enhanced reactive oxygen species production in *SDH* mutated cells (Pollard & Tomlinson, 2005; King et al, 2006).

Cellular factors associated with oxidative stress

3.4 Hypoxia

Solid tumors exhibit intratumor hypoxic states, where regions of low oxygen (hypoxia) and necrosis is common (Semenza, 2002; Maxwell et al, 1997). Hypoxia sensing and related signaling events, including activation of hypoxia-inducible factor 1 (HIF-1) now suggest that NAD(P)H oxidases, Nox1 and Nox4 serve as oxygen sensors. The human Nox4 promoter harbors putative hypoxia responsive element (HRE), which binds hypoxia-inducible factor-1 alpha (HIF-1 α) (Diebold et al, 2010). Similarly, Nox1 mRNA and protein expression is enhanced in lung cells exposed to hypoxia (Goyal et al, 2004). Hypoxia-induced activation of Nox1-dependent reactive oxygen species generation was necessary for activation of HIF-1-dependent gene expression, which was blocked by the anti-oxidant, catalase (Goyal et al, 2004). In support of these conclusions, Nox1 and Nox4 are increased by chronic exposure of mice to hypoxia (Mittal et al, 2007). In RCC, the biological significance of hypoxia-induced Nox4 and Nox1 is unclear but may mediate HIF- and NF- κ B-dependent signaling. In endothelial cells exposed to hypoxic conditions, superoxide is formed at the ubiquinone site of complex III in the mitochondria (Chandel et al, 2000). However, it is unclear if mitochondria participate in hypoxia-induced reactive oxygen species generation in renal cell carcinoma.

3.5 Growth factors

Stabilization of HIF-alpha binds to the HIF-beta subunit (ARNT) and the dimer translocates to the nucleus and binds to HIF-responsive elements, HREs (core sequence of 5'-RCGTG-3' in the enhancer elements of target genes) which drives the transcriptional activation of over a hundred genes that support renal carcinogenesis including, but limited to, vascular endothelial growth factor (VEGF) and platelet-derived growth factor-beta (PDGF-b), implicated in angiogenesis and transforming growth factor alpha (TGF-a), which can establish a mitogenic autocrine loop with the epidermal growth factor (EGF) receptor (EGFR) (Knebelmann et al, 1998; Maxwell & van den Berg, 1999; de Paulsen et al, 2001) in renal epithelial cells. The growth factors bind to their respective receptors (VEGF-R, PDGF-R and EGF-R), which are each tyrosine kinase receptors. Growth factor-induced redox signaling by Nox oxidases is well established and involves several redox-sensitive steps. Activation of signaling pathways, mediated by the aforementioned tyrosine kinases, requires inactivation of a large family of enzymes that dephosphorylate tyrosine residues, protein tyrosine phosphatases (PTPs). All PTPs contain an essential cysteine residue, which is highly susceptible to oxidation by reactive oxygen species, especially by hydrogen peroxide, leading to reversible inhibition (Rhee et al, 2003; Chiarugi & Cirri, 2003; Lee et al, 1998). By inhibiting the activity of PTPs, NADPH oxidase derived reactive oxygen species can affect the activity of tyrosine kinase signaling pathways. For example, Nox4 has been implicated in modulating PDGF-induced cell growth, VEGF-induced angiogenic responses, insulin induced glucose uptake, and insulin-like growth factor-1-induced antiapoptotic effects, although in different types of cells (Mahadev et al, 2004; Datla et al, 2007; Wagner et al, 2007). Another kinase activated in the majority of RCCs is the phosphatidylinositol 3-kinase (PI3K). PI3K signaling is regulated by the tumor suppressor phosphatase and tensin

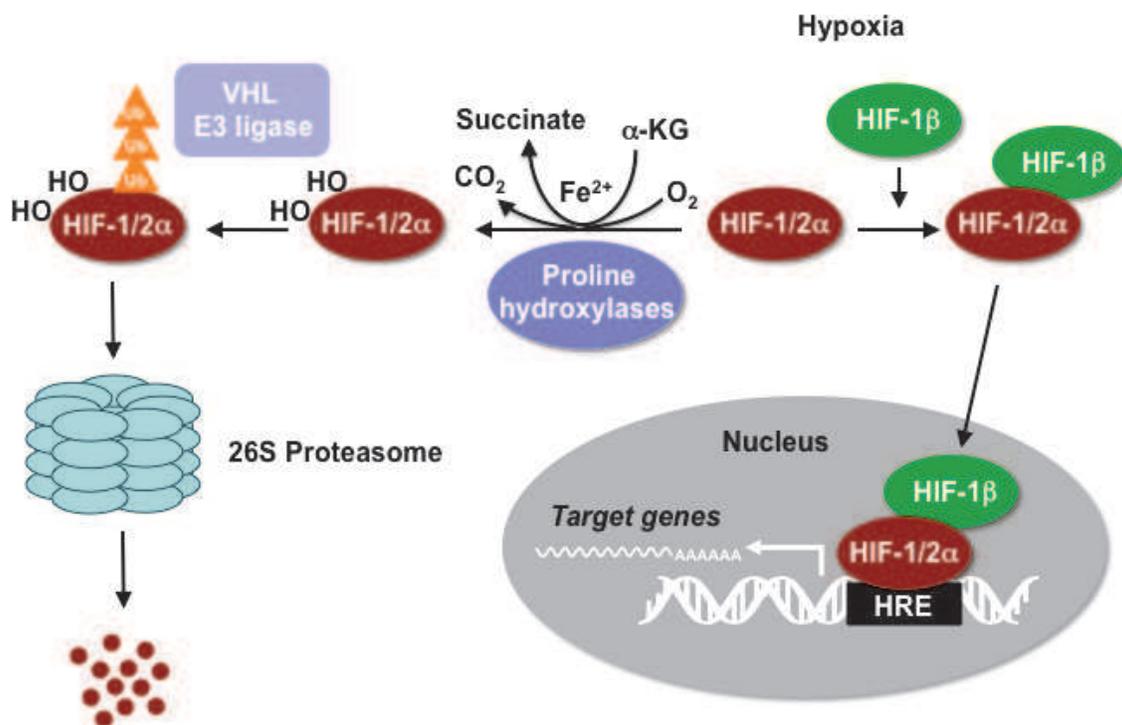


Fig. 4. Regulation of Hypoxia Inducible Factors (HIFs).

homolog (PTEN). PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate, a product of the PI3 kinase (PI3K) reaction. In various cell types, overexpression of the Nox catalytic subunit, Nox1, potentiates PIP3 generation and activation of the protein kinase Akt induced by EGF, PDGF, and insulin as a result of hydrogen peroxide-dependent oxidation of essential cysteine residue of PTEN (Cho et al, 2004; Mahadev et al, 2004). Mutations in PTEN, although common in a number of cancers, are not commonly detected in RCC. However, reactive oxygen species -induced inactivation of PTEN has not been examined in RCC.

4. Redox-signaling in renal cancer

4.1 Redox regulation of hypoxia inducible factors (HIFs)

A common endpoint in the majority of RCC, independent of histological type, is the stabilization of HIF- α subunits through multi-step processes regulated at several levels by redox-sensitive pathways. HIF- α contains two highly conserved proline residues, located at the NH₂-terminus in the oxygen-dependent degradation domains (ODDs), which are modified by a family of 4-prolyl hydroxylases (Epstein et al, 2001; Bruick & McKnight, 2001). Proline hydroxylases (PHDs) catalyze the hydroxylation reaction, which requires oxygen and 2-oxoglutarate (2-OG) as substrates and iron and ascorbate as cofactors. Proline hydroxylation promotes HIF- α binding to the multimeric VHL E3 ubiquitin ligase complex (Kamura et al, 1999). When hydroxylated and bound to VHL, HIF- α is polyubiquitinated and targeted for regulated protein degradation through the 26S proteasome (Jaakkola et al, 2001; Maxwell et al, 1999). HIF- α can also be hydroxylated at the COOH-terminus by asparaginyl hydroxylases which are Fe(II)- and 2-oxoglutarate (2-OG)-dependent family of dioxygenases (Masson & Ratcliffe 2003; Lando et al, 2002). Asparagine hydroxylation silences the COOH-terminal transactivation domains of HIF- α by preventing their interaction with the p300/CBP coactivator (Mahon et al, 2001). Reactive oxygen species inhibit PHD activity by oxidizing the PHD cofactors ferrous iron (Fe²⁺) to Fe³⁺ (Gerald et al, 2004). In solid VHL-competent tumors (hypoxic conditions), where mTOR is inactivated, reactive oxygen species are enhanced, likely stabilizing HIF- α subunits by inactivation of PHDs. Inactivation or loss of Fumarate Hydratase (FH) or Succinate dehydrogenase (SDH) can also lead to the inactivation of PHDs through different mechanisms. In FH-deficient cells, fumarate can competitively inhibit 2-OG-dependent HIF-hydroxylation resulting in the escape of VHL-dependent degradation (O'Flaherty et al, 2010), providing a VHL independent mechanism for dysregulation of HIF expression. Mutations in the Succinate dehydrogenase (*SHD*) gene promote the accumulation of succinate. Succinate is one of the end products of prolyl hydroxylase activity. Thus, succinate accumulation can block prolyl hydroxylase function and cause an accumulation of HIF- α (Pollard et al, 2005). In summary, loss of FH or SDH plays a role in HIF- α stabilization through inhibition of PHDs through metabolites and likely not through reactive oxygen species. Whereas, when VHL is mutated, expression of HIF- α subunits is maintained through Nox-dependent redox-sensitive pathways that mediate ongoing mRNA translation, discussed below (Block et al, 2007).

4.2 Redox regulation of PI3K-Akt signaling

The PI3K/Akt/mTOR signaling pathway is activated in the majority of renal cell carcinomas and mediates biological outputs such as cell growth, cell proliferation,

metabolism, and cell survival (Manning & Cantley, 2007; Porta & Figlin, 2009). The PtdIns(3,4,5)P3 phosphatase PTEN that blocks PI3-kinase signaling is mutated in ~30% of renal cell carcinomas. Reactive oxygen species -dependent inactivation of PTEN in renal cell carcinoma has not been studied, but likely to occur. When PI3-kinase is activated, protein kinase B (Akt) and phosphoinositide-dependent protein kinase 1 (PDK1) translocate to the membrane and binds to PtdIns(3,4,5)P3 and PtdIns(3,4)P2 through the pleckstrin domain (Franke et al, 1997). The colocalization of activated PDK1 and Akt allows Akt to be phosphorylated by PDK1 on threonine 308, leading to partial activation of Akt. Full activation of Akt occurs upon phosphorylation of serine 473 by a Rictor-associated mTORC2 complex (see below). There are three isoforms of serine/threonine Akt in humans; Akt1, Akt2, and Akt3. In renal cell carcinoma, the Akt2 isoform maintains HIF-alpha expression in the absence of VHL (Toschi et al, 2008). The PI3K/Akt signaling pathway is regulated by reactive oxygen species produced by p22phox-based Nox oxidases (Block et al, 2007). Furthermore, the catalytic subunit implicated in reactive oxygen species-dependent Akt activation appears to be Nox1 and Nox4 (Block et al, 2007). Treatment of renal carcinoma cells with the PI3K inhibitor, LY29002 or wortmannin has no effect on Nox activity, suggesting Nox-derived reactive oxygen species act as an upstream regulator of PI3K/Akt signaling cascade (Block et al, 2007).

4.3 Redox regulation of mTOR signaling

Translational control of existing mRNAs allows for quick changes in cellular concentrations of encoded proteins. Regulation of the rate of translation is complex and occurs at several steps. One major step of translational regulation occurs at the cap-recognition stage. This is controlled by the formation of the eIF4F complex, which include the cap-binding factor eukaryotic translation initiation factor 4E, eIF4E, and its binding partners, eIF4G and the RNA helicase, eIF4A. Binding of eIF4F complex to the mRNA cap structure is inhibited by eIF4E-binding protein 1, 4E-BP1 (Gingras et al, 2004). 4E-BP1 competes with eIF4G for a common binding site within eIF4E (Marcotrigiano et al, 1999). Therefore, when eIF4E is bound to 4E-BP1, cap-dependent translation is inhibited. Release of 4E-BP1 from heterodimerization with eIF4E is regulated by mammalian target of rapamycin (mTOR)-dependent phosphorylation of 4E-BP1. Activation of mTOR is controlled by upstream kinases known to be constitutively active in most renal cancers, the PI3K/Akt- and the RAS/MAPK-signaling pathways. The regulation of the PI3K/Akt/mTOR signaling is redox-sensitive and is regulated by p22phox-based Nox oxidases (Block et al, 2007, 2009). The Nox catalytic isoforms, Nox1 and Nox4 play an important role in stabilizing/maintaining HIF-alpha protein expression in the absence of VHL through an Akt-mTOR signaling mRNA translational pathway. As previously discussed, activation of Akt signaling is likely mediated, in part, through inactivation of the PI3K-dependent phosphatase, PTEN. The hamartin/tuberin (TSC1/TSC2) complex is an upstream negative regulator of mammalian target of rapamycin complex 1 (mTORC1). Activation of Akt leads to Akt-mediated phosphorylation of TSC2 at amino acid, T1462, which leads to TSC2 dissociation from TSC1 and is targeted for regulated protein degradation through the 26S proteasome (Plas & Thompson, 2003). Post-translational inactivation of tuberin/TSC2 has been identified in conventional clear cell renal carcinoma, which exhibits hyperactive Akt signaling (Block et al, 2010). In cultured and human RCC where p22phox and Nox-derived reactive oxygen species are high, protein expression of TSC2 is significantly reduced due to

Akt-dependent phosphorylation and degradation. Activation of mTOR, in turn, phosphorylates, several substrates necessary to activate mRNA translation. Phosphorylation of 4E-BP1 results in its dissociation from eIF4E. mTOR-dependent phosphorylation of S6K leads to its activation and downstream phosphorylation of other proteins, which collectively affect translation initiation and elongation (Holz et al, 2005; Yang et al, 2003). The RAS/MAPK pathway mediates translation by phosphorylation of translational elongation factors, including eIF4E. eIF4E is a bonafide oncogene, that when activated, through phosphorylation, inhibits its binding to the translational repressor 4E-BP1 leading to aberrant and unregulated ongoing mRNA translation of oncogenes (Mamane et al, 2004). Therefore, eIF4E is considered an oncogene involved in cell cycle progression, cell transformation, and cell survival. Misregulation of mRNA translation and constitutive activation of mTOR contributes to renal cancer. mTOR is the catalytic subunit of two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 and mTORC2 are part of a multimeric complex commonly referred to as the Raptor-associated mTORC1 (Rapamycin sensitive), and Rictor-associated mTORC2 (rapamycin-insensitive). Raptor and Rictor are scaffolding proteins within each complex allowing the assembly of other proteins. mTORC1 complex consists of mTOR, Raptor, mammalian LST8/G-protein β -subunit like protein (mLST8/G β L), PRAS40 and Deptor (Kim et al, 2002, 2003; Harris & Lawrence, 2003). mTORC2 complex consists of mTOR, Rictor, G β L, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) (Sarbasov et al, 2004, 2005; Frias et al, 2006), Protor and Deptor. Unlike mTORC1, the regulation and downstream substrates of mTORC2 are less understood. mTORC2 phosphorylates AGC kinases such as the serine/threonine protein kinase Akt, at the hydrophobic motif (HM) site, Ser473 in the presence of growth factors, which is enhanced by PI3K activity. Rapamycin, a natural inhibitor of mTOR signaling, binds the FK506-binding protein (FKBP12) and, in turn, rapamycin-FKBP12 binds mTOR inhibiting phosphorylation of raptor-associated mTOR (mTORC1) substrates, but not rictor-associated mTOR (mTORC2) substrates. Prolonged treatment and higher dosage of rapamycin has been reported to inhibit mTORC2 in a subset of cell lines. Rapamycin analogues (mTORC1 inhibitors) have been utilized for the treatment for RCC. Despite initial excitement, objective response rates to these drugs remain low. One reason for rapamycin resistance of RCC may be due to absent and/or incomplete mTORC2 inhibition. In support of these clinical findings, *in vitro* studies have demonstrated that shRNA-mediated knockdown of Rictor (TORC2 complex) but not Raptor (TORC1 complex) reduces HIF-2 α protein expression, suggesting TORC2 signals through yet unidentified pathways involved in mRNA translation to maintain HIF-2 α protein expression (Toschi et al, 2008). A role for mTORC2 in mRNA translation is now becoming evident. mTORC2 complex has been found to associate with ribosomes in a PI3K-dependent manner and phosphorylates nascent Akt at the turn motif (TM; Thr450) site, which is not inducible by growth factors (Oh et al, 2010). In cancer cells, including renal cancer, where PI3K is constitutively active, mTORC2 binding to the ribosomes is enhanced. Additionally, it has been demonstrated that treatment of some cancer cell lines with rapamycin and rapalogs increase Akt and eIF4E phosphorylation involving PI3K and Mnk kinases (Wang et al, 2007, 2008). Importantly, cultured VHL-deficient cell lines and RCC cell lines cultured from patient tumors exhibit this phenomenon. Together, this provides an alternative mechanism for rapamycin resistance of RCC and may explain why so few patients respond to rapalog therapy.

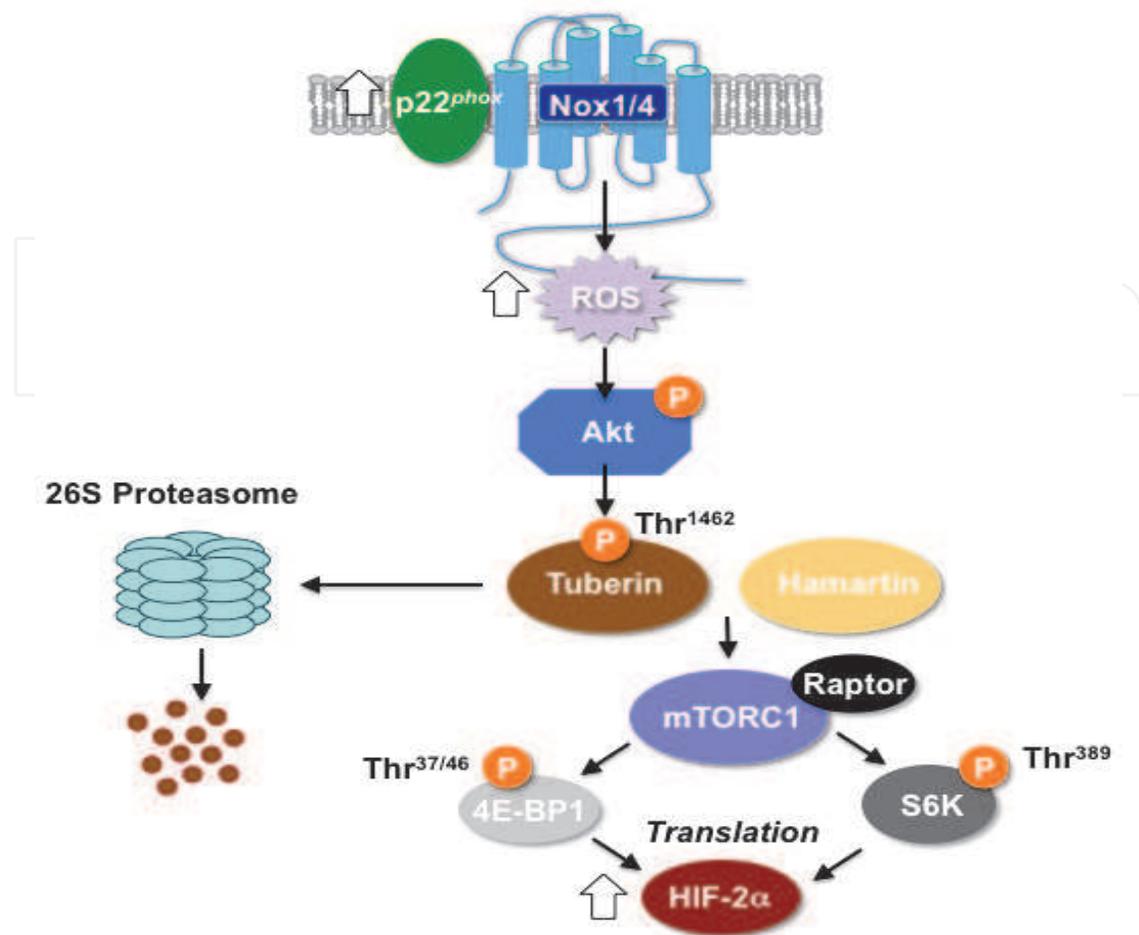


Fig. 5. Proposed mechanism of reactive oxygen species derived from p22phox-dependent Nox oxidases in the regulation of HIF-2 α mRNA translation in RCC.

4.4 Redox regulation of nuclear factor kappa B (NF- κ B) signaling

Nuclear factor kappa B (NF- κ B) is a family of redox-sensitive dimeric transcription factors that regulate hundreds of genes involved in inflammation, proliferation, angiogenesis, and cell survival (Pande & Ramos, 2005). NF κ B is constitutively expressed in a number of cancers, including renal cancer. It has been proposed that the resistance of RCC to chemotherapy and radiotherapy is due to increased levels of the nuclear factor κ B activity and resistance to apoptosis (Oya et al, 2001; Qi & Ohh, 2003). In an unstimulated state, NF- κ B binds a member of the inhibitory (I κ B) family in the cytoplasm. Activation of NF- κ B occurs in response to a wide variety of extracellular stimuli resulting in I κ B phosphorylation and subsequent regulated protein degradation. The dissociation of I κ B unmasks the NF- κ B nuclear localization sequence allowing NF- κ B to localize to into the nucleus where it heterodimerizes with a member of the NF- κ B/Rel/Dorsal (NRD) family of proteins (Pande & Ramos, 2005). Although there are five known NRD members, RelA, cRel, RelB, p50 and p65, the classical dimer is composed of p50 and RelA. Reactive oxygen species have been implicated as second messengers involved in the activation of NF- κ B as several studies have demonstrated that activation of NF- κ B by nearly all stimuli can be blocked by antioxidants (Schulze-Osthoff et al, 1997, 1998; Giri & Aggarwal, 1998). Reactive oxygen species on NF- κ B activation is further supported by studies demonstrating that hydrogen peroxide induces

NF- κ B-dependent interleukin-8 expression in endothelial cells, which contributes to the angiogenic phenotype (Shono et al, 1996). Nox oxidase catalytic subunits, Nox1 and Nox4 have been implicated in the activation of NF- κ B. Although the mechanisms remain to be determined, it is likely through regulation of I κ K phosphorylation and degradation (Dröge, 2002). More recent studies suggest that NF- κ B upregulates Nox oxidase expression and production of Nox-dependent reactive oxygen species. Here, overexpression of p65/RelA or IKK β up-regulated Nox1, Nox4, and p22phox, mRNA, and protein expression through direct binding of the respective promoters (Manea, et al 2007, 2010). In contrast, NADPH-dependent superoxide production (Nox activity) was reduced in the presence of NF- κ B inhibitors. Together, this suggests that NF- κ B acts upstream to mediate Nox-dependent reactive oxygen species production and downstream NF- κ B activity is positively regulated by Nox-generated reactive oxygen species.

5. Cell growth, survival and metastatic pathways regulated by redox-signaling in renal cancer

5.1 Mitochondrial-derived reactive oxygen species as a mediator of cell proliferation

Cancer cells utilize aerobic glycolysis and glutamine metabolism to generate the necessary resources for rapid cell proliferation and anchorage-independent cell growth. Altered glucose metabolism in cancer cells is termed the Warburg effect, which describes the propensity for most cancer cells to take up glucose avidly and convert it primarily to lactate, despite available oxygen (aerobic glycolysis) (Warburg et al, 1924). In addition to enhanced glucose metabolism, cancer cells also depend on continued mitochondrial function for metabolism, specifically glutaminolysis or glutamine metabolism. Glutamine's importance in tumor cell metabolism derives from characteristics it shares with glucose. The glutamine-fueled TCA cycle leads to the generation of reactive oxygen species by mitochondrial complexes of the electron transport chain and results in generation of ATP, NADPH, amino acids, nucleotides, and lipids (Wise et al, 2008; DeBerardinis, 2008). Mitochondrial metabolism of glutamine is elevated in cancer cells and the type of oncogenes activated in the tumor cells influences glutamine metabolism. For example, tumor cells that exhibit K-ras activation results in enhanced glutamine metabolism, fueling mitochondrial metabolism and mitochondrial derived reactive oxygen species-generation through complex III, independent of OXPHOS, which is necessary for cellular proliferation and anchorage-independent cell growth (Chandel et al, 2000). Additionally, c-Myc enhances glutamine metabolism in cancer cells by enhancing glutaminase (GLS), an amidohydrolase enzyme, which generates glutamate from glutamine. In prostate cancer cells, GLS is important for Myc-induced cell proliferation. K-ras and c-Myc amplification has been detected in RCC. Deciphering the pathways that fuel the TCA cycle differentially in renal cancer cells of various histologies will be important to elucidate the role of mitochondria in RCC cell proliferation and anchorage-independent growth.

5.2 Nox oxidase-derived reactive oxygen species as a mediator of cell proliferation

In renal cell carcinoma, inhibition of Nox oxidases using the NAD(P)H oxidase flavoprotein inhibitor diphenylene iodonium, DPI, reduces cell number and tumor growth in a xenograft nude mouse model; however, the mechanisms by which Nox-derived reactive oxygen

species mediate cell proliferation remain unclear (Block et al, 2007). Kidney cancers demonstrate enhanced activation of redox-sensitive signaling pathways involved in cell proliferation. Notably, HIF-2 α , rather than HIF-1 α , has been shown to play a critical role in renal tumorigenesis due to HIF-2 α driven TGF- α expression, the mitogen for proximal tubular epithelial cells. Up-regulation of TGF- α leads to its binding to the epidermal growth factor receptor (EGFR) with subsequent activation of the PI3K/Akt signaling pathway. As discussed earlier, growing evidence suggest that Nox-derived reactive oxygen species can stimulate signal transduction cascades through the EGFR likely through protein tyrosine phosphatase (PTP) inhibition. A role for Nox oxidases in agonist-induced cell proliferation has been demonstrated in a variety of other cell types; for example, proliferating keratinocytes showed higher reactive oxygen species generation and Nox1 expression than quiescent cells (Chamulitrat et al, 2003). Over-expression of Nox1 in several cell types is associated with increased cell division (Suh et al, 1999; Ranjan et al, 2006; Kamata et al, 2005). In addition, Nox overexpression has been seen in human renal, colon, prostate cancers and melanomas. In the case of Nox4 in melanoma cells and Nox5 in prostate cancer cells, inhibition of reactive oxygen species resulted in inhibition of cell proliferation, supporting a role for reactive oxygen species in mitogenic signaling (Lassegue & Clempus, 2003).

5.3 Reactive oxygen species as a mediator of cell survival

Increased reactive oxygen species is normally linked to cell death. However, in a subset of cancers, Nox-dependent reactive oxygen species has been associated with cell survival. For example, Nox4- and Nox1-derived reactive oxygen species inhibits apoptosis in pancreatic cancer cells and colon cancer cells respectively in a NF kappa-B- (Fukuyama et al, 2005) and Akt-dependent manner (Mochizuki et al, 2006). It is still unknown what role Nox oxidases and/or mitochondrial-derived reactive oxygen species play in RCC cell survival.

5.4 Reactive oxygen species as a mediator of angiogenesis

Renal tumors are known to be a highly vascular due to enhanced angiogenesis. Angiogenesis is the process in which tissue recruits blood vessels to form a neovasculature to vascularize the tissue. In most cases, the intratumor tissue experiences physiologic hypoxia and generates the angiogenic growth factor vascular endothelial growth factor (VEGF). VEGF induces angiogenesis by stimulating endothelial cell proliferation and migration primarily through the receptor tyrosine kinase VEGF receptor-2. VEGF binding initiates tyrosine phosphorylation of KDR, which results in activation of downstream signaling enzymes including ERK, Akt and eNOS, which contribute to angiogenic-related responses in endothelial cells (Colavitti et al, 2002; Matsumoto & Claesson-Welsh, 2001). Although NADPH oxidases are important for maintaining HIF- α expression in RCC, it is likely that Nox oxidases play a broader role in angiogenesis. Nox-derived reactive oxygen species function as signaling molecules to mediate various angiogenic-related responses such as cell proliferation, migration and angiogenic gene expression in endothelial cells (Ushio-Fukai et al, 2002, 2004, 2006). In endothelial cells, NADPH oxidase is activated by numerous stimuli including VEGF, EGF, cytokines, and hypoxia. Downregulation of Nox4 inhibits VEGF-induced endothelial cell migration and proliferation (Datla et al, 2007). Nox4

expression is upregulated in new capillaries in brain ischemia-induced angiogenesis of mice (Vallet et al, 2005). In animals of prostate cancer, Nox1 over-expression markedly increased angiogenesis by inducing the angiogenic factor VEGF correlating with an aggressive tumor phenotype (Lim et al, 2005). Nox1-induced hydrogen peroxide increases VEGF and VEGF receptor expression and MMP activity, markers of the angiogenic switch, thereby promoting vascularization and rapid expansion of melanoma tumors (Arbiser et al, 2002). Nox2 generates reactive oxygen species in endothelial cells by a number of agonists including VEGF and Ang 1, which are involved in angiogenesis (Ushio-Fukai et al, 2002; Gorchach et al, 2000; Li & Shah, 2002; Frey et al, 2002; Fürst et al., 2005; Harfouche et al, 2005). Neovascularization in response to ischemia or VEGF is inhibited in Nox2^{-/-} mice and in wild-type mice treated with a NADPH oxidase inhibitor (Ushio-Fukai et al, 2002; Tojo et al, 2005; Al-Shabrawey et al, 2005). Taken together, accumulating evidence suggest that reactive oxygen species derived from NADPH oxidases play an important role in physiological and pathological angiogenesis; however, the enzymatic sources and role of reactive oxygen species involved in renal cancer angiogenesis remain undetermined.

6. Oxidative stress as potential novel biomarkers or therapeutic treatments in renal cancer

6.1 Novel biomarkers

Metabolites are the intermediates and products of metabolism. Whether its mitochondrial dysfunction, mutation in TCA cycle genes, or abnormal oxygen consumption, metabolic profiling can provide a metabolite fingerprint of intracellular physiology within a tumor. As the kidney is an organ, which secretes the water and waste drain from each kidney to the bladder and are eliminated from the body as urine, small-molecule metabolites are likely to be found in the urine. Metabolic profiling may be used for the establishment of non-invasive urinary biomarkers for the prediction of renal cancer, prognostic indicator, or responsiveness to therapy. A comprehensive metabolomics-driven approach is needed for the identification of biomarkers in various histologies of RCC. The most representative product that may reflect oxidative damage induced by reactive oxygen species detectable in the urine is 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Sakano et al, 2009). F2-Isoprostanes and malondialdehyde (MDA) are considered reliable markers of lipid peroxidation *in vivo* and can also be detected in the urine. However, the use of oxidative stress markers as biomarkers for RCC may be challenging as many co-morbidities such as diabetes and hypertension induce oxidative stress that may be detected in the urine.

6.2 Antioxidants

The use of antioxidants to prevent disease is controversial. Antioxidants are manufactured within the body and are naturally found in fruits and vegetable food sources. As this chapter has just revealed a broad role for reactive oxygen species in renal tumorigenesis, it would be rational to think that antioxidants will slow or prevent activation of oncogene signaling in tumor cells. Indeed, *in vitro* studies demonstrate some beneficial effects of antioxidants on tumor cells and observational studies suggested a diet high in fruits and vegetables, both of which are rich with antioxidants, may prevent cancer development. However, many randomized trials have indicated that there is no benefit in preventing

cancer or affecting mortality with antioxidant supplementation using vitamin C, vitamin E, or beta carotene in human patients (Lin et al, 2009). Supplementation with vitamin C, along with vitamins A, E, and beta-carotene did not prevent gastrointestinal cancer (Bjelakovic et al, 2004) did not lower the risk of prostate cancer (Kirsh et al, 2006) however, one study did find an association between the intake of vitamins A, C, or E and a reduced risk for cervical cancer (Kim et al, 2010). Are the successes or failure of antioxidants organ or genetic specific? All cells have intracellular antioxidant defense systems. However, as discussed, neutralization of free radicals are not 100% efficient and some proteins that function to neutralize the antioxidants are significantly reduced or inactivated in cancers, including renal cancer. Moreover, the enzymatic sources that generate reactive oxygen species are overactive and are not "turned off" by antioxidants. Here, it is likely that co-morbidities such as diabetes and hypertension play a systemic biological role in antioxidant failures, as diabetes and hypertension are known to induce oxidative stress alone, without the compounding issues of a tumor and tumor environment. Taken together, it is evident that a successful approach for antioxidant therapy will be to target the enzymatic sources that produce the reactive oxygen species such as NADPH oxidases or the mitochondria. Targeting Nox enzymes in an isoform-selective manner is likely to offer therapeutic advantages.

6.3 Novel therapeutic targets

Hypoxia inducible factors are master transcriptional regulators that activate over 100 genes involved in renal tumorigenesis. Therefore, targeting HIF-alpha subunits is an attractive therapeutic clinical goal. To date, agents with anti-angiogenic activity that inhibit VEGFR and PDGFR signaling (e.g. sorafenib, sunitinib), the VEGF ligand (bevacizumab), and the EGF ligand (cetuximab) have demonstrated some effectiveness in the management of renal cell cancer to different degrees (Patel et al, 2006; Sosman et al, 2007). However, these agents target only a small portion of the downstream genes regulated by HIF. As outlined here, the majority of renal cancer exhibits stabilization of HIF-alpha through the loss of VHL function or inhibition of proline hydroxylation activity together resulting in HIF-alpha overexpression. In the absence of VHL, maintaining HIF-alpha expression is dependent on ongoing mRNA translation, regulated by mTOR signaling. However, clinical trials using approved mTOR inhibitors such as temsirolimus (CCI-779) and everolimus (RAD001) do not exhibit beneficial outcomes long term. Importantly, renal carcinoma cells express HIF-2alpha or HIF-1alpha/HIF-2alpha and knockout and molecular studies have revealed that HIF-1alpha translation is dependent on mTORC1 signaling, whereas HIF-2alpha is downstream of the mTORC2 pathway; therefore, rapalogs have little to no effect on reducing HIF-2alpha expression in renal cell carcinoma (Toschi et al, 2008). Because Nox-dependent reactive oxygen species production maintain HIF-2alpha in the absence of proteasomal degradation by VHL and the broader role Nox oxidases play in other signaling pathways that mediate the genesis of RCC, suggest that novel development of specific inhibitors of NADPH oxidases may provide a novel approach for therapeutic targeting. For now, based on the literature and molecular mechanisms of mTOR signaling, new agents that target both the mTORC1 and mTORC2 pathways have the potential to downregulate both HIF-1alpha and HIF-2alpha in clear cell kidney cancers and could provide more antitumor activity than temsirolimus and everolimus, which again primarily target the mTORC1

pathway. Agents, which inhibit mTORC1, mTORC2 and PI3K pathways, such as AZD8055, demonstrates potent anti-tumor activity in *in vitro* and *in vivo* model systems (Chresta et al, 2010). It is unclear, if these inhibitors have indirect antioxidant effects.

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8. References

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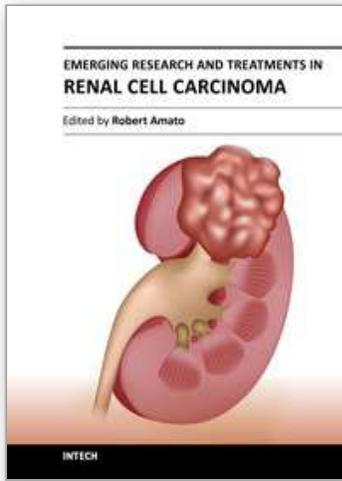
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