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Redox signaling at invasive microdomains in cancer cells

Begoña Díaz and **Sara A. Courtneidge***
Cancer Center, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA.

Abstract

Redox signaling contributes to the regulation of cancer cell proliferation, survival and invasion, and participates in the adaptation of cancer cells to their microenvironment. NADPH oxidases are important mediators of redox signaling in normal and cancer cells. Redox signal specificity in normal cells is in part achieved by targeting enzymes that generate reactive oxygen species to specific subcellular microdomains such as focal adhesions, dorsal ruffles, lipid rafts or caveolae. In a similar fashion, redox signal specificity during cancer cell invasion can be regulated by targeting reactive oxygen generation to invasive microdomains such as invadopodia. Here we summarize recent advances in the understanding of the redox signaling processes that control the cancer cell pro-invasive program by modulating cell adhesion, migration and proteolysis as well as the interaction of cancer cells with the tumor microenvironment. We will focus on redox signaling events mediated by invadopodia NADPH oxidase complexes and their contribution to cancer cell invasion.

Keywords

Redox; ROS; signaling; cancer; migration; invasion; invadopodia; NADPH oxidase; Tks4; Tks5; Nox4; Nox1; p22phox; PTPs

INTRODUCTION

Reactive oxygen species (ROS) are mediators of redox signaling and oxidative stress, two distinct but related processes which contribute to neoplasia. Redox signaling is initiated by physiologically generated ROS to regulate cellular functions or decisions. ROS act as second messengers due to their ability to induce covalent modification (oxidation) of biological macromolecules which affects their functions in a similar way to phosphorylation [1]. Redox signaling pathways are used by normal and cancer cells to modulate physiological or aberrant cellular functions, respectively. Oxidative stress can be initiated by the unregulated production of ROS from either extracellular (ultraviolet irradiation, drugs, xenobiotics) or intracellular (mitochondria, peroxisomes, oncogenes) sources [2]. These ROS are usually present at high levels and can cause damage by irreversibly oxidizing cellular proteins, lipids and nucleic acids [3]. In normal cells oxidative stress elicits an anti-oxidant response that leads to either damage repair or death; however cancer cells aberrantly tolerate oxidative stress [4–7] which contributes to cancer progression by driving, for instance, genetic instability [8–10]. The physiological induction of antioxidant enzyme expression in response to oxidative stress is also considered by some investigators as a process of redox signaling [11]. Despite the differences between both processes, oxidative stress and redox signaling are far from being independent events, especially in cancer, where ROS derived from oxidative stress could potentially activate a redox signaling pathway, or a

*corresponding author, courtneidge@sbmri.org, phone: +1 858 646 3128.

deregulated redox signaling pathway could contribute to oxidative stress by virtue of excess ROS generation. We are still far from understanding how any cell senses and integrates inputs from different ROS signals, whether dangerous or beneficial for tissue homeostasis.

Oxidative stress and redox signaling have been implicated in the initiation and/or maintenance of a pro-invasive program in cancer cells. We will focus here on recent advances in redox control of cellular signaling pathways during cancer cell invasion and, in particular, on novel redox signaling complexes which localize ROS to subcellular microdomains (invadopodia) in order to drive cell invasion.

OVERVIEW OF REDOX SIGNALING IN CANCER

Basic components of redox signaling

The generation of ROS during redox signaling is enzymatically regulated. The main sources of ROS are cellular oxidases, [12] including xanthine oxidases [13], lipoxygenases [14, 15], cyclooxygenases [16], myeloperoxidases [17] and NADPH oxidases [18]. Additionally, lysyl oxidases are amine oxidases which directly oxidize extra and intracellular substrates [19, 20]. Partial one electron reduction at mitochondrial respiratory chain complexes 1 and 3 also contributes to the generation of ROS [21], although their role in redox signaling as opposed to oxidative stress is less well understood.

NADPH oxidases are among the best-characterized enzymes that generate ROS for cellular signaling purposes [18, 22, 23]. There are seven Nox family members: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2. All are transmembrane flavoproteins capable of generating superoxide by transferring an electron from NADPH to molecular oxygen. They contain six transmembrane alpha helical domains and an extracellular domain which can be glycosylated if the Nox subunit is localized at the plasma membrane [24]. Some NADPH oxidases localize to other cellular membranes, including endoplasmic reticulum [25] and intracellular vesicles [26].

With the possible exception of the calcium-regulated family member Nox5, Noxes require additional subunits for maximal oxidase activity. Nox1, Nox2, Nox3 and Nox4 bind to the transmembrane protein p22phox [27], which in turn recruits organizers (p47phox, p40phox or NoxO1), activators (p67phox or NoxA1) and small GTPases (Rac1 or Rac2) [28–32]. In the case of Nox2 for example, the SH3 domains of p47phox associate with the C-terminus of p22phox while the proline-rich tail of p47phox associates with p67phox, which in turn is associated with active Rac2. In over-expression experiments, Nox4-p22phox seems to behave as a constitutively active enzyme with full enzymatic activity in the absence of organizers or activators [33], although that remains to be demonstrated for the endogenous protein. In the case of Duox1 and Duox2, protein maturation and plasma membrane localization require the function of the accessory proteins DuoxA1 and DuoxA2 [34] that also seem to contribute to Duox activity at the plasma membrane [35, 36].

Superoxide generated by NADPH oxidases is converted to hydrogen peroxide in a process known as dismutation, either spontaneously at low pH, or with higher efficiency by the enzymatic activity of superoxide dismutases (SODs), which are present at different subcellular locations [37]. Three SODs have been described: SOD1 (also known as Mn-SOD) localized to mitochondria; SOD2 (also known as CuZn-SOD), localized in the nucleus and cytoplasm; and EC-SOD, which is more abundant in extracellular matrix (ECM) and fluids [38]. Hydrogen peroxide is the prominent ROS implicated in signal transduction and acts mainly through oxidation of specific cysteine and methionine residues on target molecules.

Multiple eukaryotic cellular signaling pathways use oxidation of effector molecules to transmit signals. Many of these pathways use a redox signaling module to increase the amplitude and/or duration of a tyrosine phosphorylation signal [39]. Hydrogen peroxide was first suggested to act as a signal-transducing molecule downstream of PDGF signaling in smooth muscle cells [40], but the mechanism was unknown. The discovery of transient regulation of phosphatase activity by reversible oxidation was a fundamental step in understanding the importance and extent of redox signaling [41–43]. During redox signaling, hydrogen peroxide reversibly oxidizes a conserved cysteine residue in the active site of protein tyrosine phosphatases (PTPs), inhibiting their catalytic activity [41, 44, 45]. Many studies have described the regulation of cellular processes by the transient inactivation of protein tyrosine phosphatases and dual-specificity phosphatases through redox signaling, often in the context of normal or cancer cell signaling downstream growth factor receptor or integrin activation. Examples include PTP1B [46, 47], LMW-PTP [48, 49], Shp-2 [43], PTEN [50] and PTP-PEST [51]. Amplification of tyrosine phosphorylation signals is perhaps also achieved by direct redox activation of protein kinases such as c-Src [52–54]. In addition, redox modification of many other effector molecules is relevant in normal or cancer cell signaling, including cell cycle regulators such as Cdc25C [55]; RasGTPase [56] and Ras-dependent signaling [57]; transcription factors such as c-Jun/c-Fos and Stat3 [58, 59]; matrix metalloproteases (MMPs) [60, 61]; and cytoskeletal components such as actin [62].

Novel redox signaling components: Tks family of proteins

Recently, the adaptor proteins Tks4 and Tks5 have been shown to be novel components of NADPH oxidase complexes [63, 64]. Tks5 (previously known as Fish) was first described in a cDNA library screen for Src substrates [65]. The existence of a Tks5 orthologue was suspected from information on public databases [66] and confirmed by the cloning of Tks4, which has an overall identity of 43% to Tks5 [67]. Tks4 and Tks5 are large scaffolding proteins that contain an N-terminal Phox homology (PX) domain and four or five Src homology 3 (SH3) domains, respectively [65, 67]. Both Tks family members are c-Src substrates [65, 67, 68], and several binding partners have been identified, including ADAM-family metalloproteases [69], Grb2 and N-WASP [70], dystroglycan [71], and Nck [68]. Tks proteins are expressed in different organs including brain, lung, liver, kidney, skeletal muscle and heart [65, 67]. Furthermore, Tks4 has been shown to be disrupted in patients with Frank-Ter Haar syndrome, a genetic disorder characterized by skeletal, ocular and cardiac abnormalities [72]. The Tks adaptors are expressed in several human cancer cell lines and tumors [73], and have overlapping non-redundant functions in regulating the formation and activity of podosomes/invadopodia [67, 73], although the molecular basis for these differences are still unknown.

The homology between Tks adaptors and p47phox led to the hypothesis that the Tks proteins could act as organizers for Nox-dependent ROS generation [65]. Indeed, both Tks4 and Tks5 are structurally similar to p47phox, p40phox and NoxO1: all contain amino-terminal PX domains which preferentially bind PtdIns(3,4)P2 and/or PtdIns(3)P, followed by 2 or more SH3 domains [65, 67, 74]. There is a single primordial Tks/p47 gene in tunicates and echinodermata, which is likely to be the common ancestor of the vertebrate orthologues Tks4, Tks5, p40phox, p47phox and NoxO1 [75]. The demonstration that Tks4 and Tks5 support Nox1 and Nox3-dependent generation of ROS in the absence of other organizers confirmed this hypothesis [64]. Moreover, Tks5 can associate with p22phox through a mechanism involving the first two SH3 domains of Tks5 and the proline-rich region of p22phox [63], and Tks4 and Tks5 can bind NoxA1 through their SH3 domains in a Rac-independent manner [64]. More importantly, the total levels of ROS generated by cancer cells decreases considerably after silencing of Tks5 or Tks4 [63, 64], indicating that

endogenous Tks proteins may be required for ROS generation in cancer cells. The existence of Tks-dependent ROS generation during normal cell biology remains to be proven, but it is likely that Tks proteins play an important role in redox signaling in normal tissues as well. Although in HEK293 cells the activity of the over-expressed Nox4-p22phox complex seems to be independent of adaptor or organizer proteins [33], and over-expressed Tks4 or Tks5 in these cells did not increase ROS generation by over-expressed Nox4 [64], the silencing of endogenous Tks5 decreased ROS generation by Nox4-p22phox over-expressing mouse melanoma cells [63], indicating a possible role for Tks5 in Nox4-dependent ROS generation in certain cancer cells, although this hypothesis awaits further investigation. Another PX domain-containing protein, Poldip2, has recently been shown to interact with p22phox and modulate Nox4 localization and activity in vascular smooth muscle cells [76].

Regulation of Redox signaling: how, when and where?

An excess of hydrogen peroxide is potentially dangerous; therefore its generation is tightly regulated, even in cancer cells that are known to have increased antioxidant defenses and tolerate higher ROS levels than normal cells. Regulation of ROS generation is performed at several levels: control of expression and activity of cellular oxidases, regulation of the antioxidant cellular machinery and spatial restriction of ROS generation. In addition, redox signaling is terminated by the reduction of reversibly oxidized targets.

NADPH oxidase components are expressed in different cancer cell lines and tumor tissues [77, 78]. Inducers of expression and activity of NADPH oxidases include growth factors, oncogene expression, hypoxia, as well as integrin-dependent stimulation [23, 79]. Hydrogen peroxide diffusion is limited by the cytoplasmic reducing environment, and ROS activity is restricted by cellular antioxidant defenses that include glutathione peroxidase, catalase, thioredoxin and peroxiredoxin [11, 80]. Redox signaling is terminated by the reduction of reversibly oxidized targets by cellular thiols. In the case PTP1B, this effect seems to be mediated in vitro at physiological pH by thioredoxin rather than by glutharedoxin or glutathione [46]. Which specific cellular thiols are implicated in redox signaling termination in different cellular contexts is still poorly understood.

Spatial regulation is key for the specificity and control of redox signaling pathways and has been shown to be achieved by targeting oxidants and/or limiting antioxidants to subcellular microdomains [39, 81, 82].

REDOX SIGNALING AND CANCER INVASION

Cell invasion is considered one of the limiting steps for the progression of primary solid tumors to metastatic lesions [83]. Without local invasion into neighboring tissues, the primary tumor might be resectable, but the initiation and maintenance of a cell invasive program allows the spreading of the cancer cells into the surrounding tissues and may promote metastatic disease [84]. The main cellular mechanisms implicated in the initiation and maintenance of an invasive program include modulation of the adhesive, migratory and proteolytic abilities of the cancer cell as well as its adaptation to tumor microenvironmental cues [84]. Redox signaling pathways play an important role in the regulation of all these processes.

Redox control of adhesion and migration

In cancer cells, cell-cell and cell-substrate adhesion properties change in response to pro-invasive stimulation. The modification of these adhesion complexes not only alters the adhesive and migratory properties of cells by inducing cytoskeletal remodeling, but also modulates survival and cell cycle progression by affecting the cross-talk between adhesion

platforms and the cell signaling networks that regulate the communication between cells and their microenvironment [85, 86].

In epithelial cells, cell-cell adhesion through adherens junctions is diminished by the downregulation of the cell-cell adhesion component E-cadherin. The loss of adherens junctions facilitates cell migration and it is best studied in the context of embryonic development, where the downregulation of E-cadherin in specific subsets of cells initiates the process of epithelial to mesenchymal transition (EMT) which contributes to morphogenesis [87]. In a process similar to EMT, during cancer progression epithelial cells adopt a migratory phenotype which allows the dissemination of cancer cells from their tissues of origin. Indeed, loss of E-cadherin is enough to mediate the transition from adenoma to carcinoma in mice [88]. While different mechanisms have been described to mediate the loss of E-cadherin in normal and cancer cells [89–91], ROS are necessary for EMT induced by TGFβ in renal tubular epithelial cells through activation of MAPK and Smad [92], and they promote the MMP3-dependent activation of Snail to induce EMT in SCp2 mammary carcinoma cells [93]. Furthermore lysyl oxidase-like 2 interacts with Snail and induces EMT, possibly through Snail oxidation [94].

Cell adhesion to the ECM is also modulated during invasion. Integrins and cellular adhesion molecules, or CAMs, are both implicated in cell-ECM adhesion and mediate cancer cell invasion [95, 96]. In cancer, integrin-mediated adhesion and signaling can be modulated by increased integrin recycling [97] as well as aberrant expression or activation of integrins and integrin signaling adaptors [98, 99]. Nox1 mediates anchorage-independent proliferation in vitro and subcutaneous tumor growth of K-Ras transformed cells in athymic mice [100]. This appears to be mediated by decreased cell adhesion and stress fiber formation, through inactivation of the GTPase Rho. Indeed, stress fiber formation may be abrogated by Nox1 activity through ROS-dependent transient inactivation of LMW-PTP and the consequent activation of p190RhoGAP, which in turn suppresses Rho GTPase activity [101]. Redox regulation of stress fiber formation was originally described to occur in HeLa cells, possibly through a Nox2-dependent pathway [102]. In human colon tumors, Nox1 expression correlates with K-Ras activating mutations [103], and in colon cancer cells, Nox1 dependent signaling promotes cell migration downstream of arachidonic acid and PKCs by modulating integrin recycling [104]. In bladder cancer cells, Nox1 and Nox4 regulate invasiveness through NFκB activation downstream of the leukotriene B4 receptor [105], and the activity of Nox2 and Nox4 in transformed myeloid cells increases migratory ability, perhaps by modulating the phosphorylation of MARCKS, an actin filament crosslinking protein [106]. Furthermore, ROS-dependent signaling facilitates the survival of cancer cells in the absence of attachment to the ECM. Normal cells die by anoikis in the absence of ECM-dependent signals, and this is in part due to the induction of oxidative stress. This response is abrogated by antioxidants or oncogenes that, interestingly, by protecting from oxidative stress-induced anoikis facilitate cancer progression [6]. Furthermore, Nox1 mediates the activation of an Akt-dependent survival pathway initiated by the binding of angiopoietin-like 4 protein to integrins in the absence of ECM attachment, allowing cancer cells to escape anoikis by mimicking ECM-integrin signaling [107].

Redox control of proteolysis and ECM remodeling

In some instances, invasive cells have the ability to reach the blood or lymphatic circulation and spread to distant sites, a process largely restricted in adult organs to immune surveillance. Invasive cells travel through different ECMs -mainly interstitial matrix and basement membranes- in order to extravasate, intravasate, and colonize target organs. To date, there is no unifying hypothesis to explain the mechanism used by cancer cells to cross these barriers, and it is likely that both proteolytic and non-proteolytic mechanisms are utilized [108, 109]. Non-proteolytic mechanisms may suffice for extravasation through

tumor vasculature with compromised integrity, or lymphatic vessels in which cell-cell junctions are sparser. Non-proteolytic mechanisms may also be sufficient to cross defective basement membranes (BMs) associated with decreased deposition or crosslinking of its components [110] or to travel through interstitial collagen [111]. Proteolytic mechanisms are better understood and often involve proteolysis of ECM components and their consequent irreversible remodeling. Matrix metalloproteases (MMPs) are prominent among ECM remodeling enzymes, and their activity is associated with normal physiology [112] as well as with cancer cell invasion and tumor progression [113–115]. NADPH oxidase-dependent ROS generation regulates expression or activation of MMPs in endothelial cells [116–118], cardiomyocytes [119], and alveolar macrophages [120]. In breast cancer cells, MMP-2 is activated by SOD1 through the generation of ROS [121], and in HT1080 sarcoma cells by a RTK-PI3K-NFkB pathway downstream of hydrogen peroxide [122]. MMP9 secretion in keratinocytes treated with the carcinogenic compound TPA seems to require NADPH oxidase activity [123]. Furthermore, the expression of mitochondrial SOD in fibrosarcoma cells increases the levels of MMP-1 and the incidence of experimental pulmonary metastasis [124], and the secretion and activation of MMP2 downstream of EGF signaling in pancreatic cancer cells is dependent on NADPH-generated ROS mediated by PI3K- and Src-dependent activation of Rac1 [125]. Although in some of these studies the cellular sources of ROS remain unclear, the findings suggest an important role of redox signaling in the regulation of extracellular matrix remodeling.

Redox signaling and the tumor microenvironment

Cancer cells are exposed to an abnormal microenvironment that is affected by, and in turn modulates, cancer cell biology. The tumor microenvironment contains a modified ECM and a stromal cell component that includes tumor-associated fibroblasts, adipocytes and immune cells. The tumor microenvironment evolves during tumor progression, becoming low in oxygen and nutrients and influencing and being modulated by angiogenesis, fibrosis and inflammation [126–128]. ROS participate in the regulation of these processes. For instance, secreted lysyl oxidases mediate the enzymatic oxidation of specific lysine residues on collagen to increase the formation of fibrillar collagen, the ligand for β1 integrin. Lysyl oxidase expression is induced by hypoxia and promotes cancer cell migration and invasion through FAK activation [129] as well as metastasis by generating a pre-metastatic niche [130]. Nox1 overexpression increases angiogenesis and the growth of prostate cancer cells injected subcutaneously in mice, in part through the up-regulation of VEGF [131]. Nox-dependent ROS induce angiogenesis and tumor growth of ovarian cancer cells through up-regulation of HIF1α and VEGF [132]. Furthermore, inhibition of NADPH oxidases reduces angiogenesis and tumor growth in mice [133].

LOCALIZING REDOX SIGNALING TO INVASIVE MICRODOMAINS

Invadopodia: cellular tools for invasion

In vitro, many invasive cancer cells have the ability to form invadopodia, specialized protrusions of the plasma membrane that concentrate adhesive proteins and proteolytic enzymes and contain a complex array of molecular components that coordinate cell adhesion, cell migration and pericellular proteolysis [134–136]. The cytoplasmic component of invadopodia contains cytoskeletal proteins such as microfilaments, microtubules and intermediate filaments [137]. Actin filaments serve as a scaffold for multiple actin-binding proteins and cellular signaling proteins, including the Nox organizers Tks5 and Tks4, which are required for invadopodia formation and function [64, 67, 69]. The plasma membrane at invadopodia contains a specialized subset of lipids [70] and transmembrane proteins, which include integrins [138], membrane matrix metalloproteases [139, 140] and, in Src-transformed fibroblasts, the NADPH oxidase subunit Nox4 [63]. Invadopodia formation and

their dynamic turnover depend on growth factor and cell adhesion signals. For instance, EGF and TGFβ can induce invadopodia formation in breast cancer cells [141, 142], and Twist, a transcription factor that drives EMT and metastasis, has been shown to regulate invadopodia formation [143].

Invadopodia are usually studied in 2-dimensional cell culture, although recent studies have analyzed them in more complex environments [137, 144]. The ability of invadopodia to regulate pericellular proteolysis is commonly assessed in vitro by growing cells on top of a fluorescently labeled ECM substrate. After processing for immunofluorescence, focal proteolysis can be visualized as “dark spots” where the labeled substrate has been digested (Figure 1). The ability to form invadopodia in vitro correlates with increased invasiveness in vitro and in vivo [73, 145]. While still awaiting a definitive demonstration of the presence of these structures in vivo, invadopodial components such as Tks5 and cortactin have been shown to be necessary for efficient tumor growth in animal models [146–148], suggesting that invadopodia mediate tumor progression in vivo.

Redox signaling and invadopodia

An additional mechanism by which redox signaling controls cancer cell invasion is through the regulation of invadopodia formation and activity [63, 64]. ROS can be detected at invadopodia using the ROS-sensitive fluorescent probe DCF-DA and live-cell imaging [63]. ROS are necessary for invadopodia formation in both mouse and human cancer cells, and a prominent source of ROS in this context might be novel NADPH oxidase complexes (Figure 2) containing Nox1 or Nox4 along with the novel NADPH organizers and invadopodia components Tks4 or Tks5 [63, 64]. These complexes might be exclusively formed at invadopodial membranes in cancer cells, since both Tks4 and Tks5 are invadopodia markers and localize preferentially to these structures [67, 69, 73].

Invadopodia membranes, as well as membrane ruffles, focal adhesions and caveolae can be considered as subcellular microdomains, specialized regions of the plasma membrane that contain specific signaling complexes and perform unique cellular functions. These structures, unlike other subcellular compartments such as endoplasmic reticulum, mitochondria or nuclei, are not separated from the rest of the cytoplasm by a physical barrier such as a cellular membrane. Therefore, the local regulation of these structures and its activity by redox signaling represents an additional challenge to signal-specificity mechanisms. NADPH oxidase complexes in the plasma membrane generate superoxide to the outer side of the membrane. Superoxide is not membrane permeable, unlike its dismutated product H2O2, which has a longer half life and is believed to cross the plasma membrane at the site of generation and locally affect susceptible targets. The radius of action of H2O2 is believed to be limited by the cytoplasmic reducing environment. How could then H2O2 reach its targets? This is a subject of intense investigation, and some clues are starting to emerge. For instance, Src activity has recently been shown to mediate redox signaling by facilitating a local increase in H2O2 concentration through the phosphorylation and transient inactivation of membrane-bound Peroxiredoxin-1 [82]. Src kinase activity promotes invadopodia formation [149, 150], and it is tempting to speculate that redox control of invadopodia formation also requires local inactivation of Peroxiredoxin-1 locally present to modulate the redox signal. At subcellular microdomains such as invadopodia, redox signal specificity is then regulated by the activation of ROS-generating enzymes and the inactivation of ROS-scavenging proteins within the limits of the microdomain.

Local accumulation of ROS at membrane sites has also been observed at membrane ruffles of endothelial cells induced to migrate using the scratch assay [39, 151, 152]. The local generation of ROS by targeting Nox to discrete subcellular membrane locations through protein-specific associations contributes to redox signaling specificity and regulates

directed-cell migration in endothelial cells (reviewed in [81]). For example, after VEGF stimulation of endothelial cells, p47phox is targeted to membrane ruffles by association with the cytoskeletal protein WAVE1, and forms a complex containing Rac1 and PAK1 that seems to modulate actin assembly and JNK activation through a ROS-dependent pathway [153]. An NADPH complex containing Nox2 and the scaffold protein IQGAP1 localizes to the lamellipodia of migrating endothelial cells and is necessary for localized ROS generation, cytoskeletal reorganization and migration [152]. The orphan adaptor TRAF4 recruits p47phox to focal complexes along with Hic-5 to activate PAK1 and drive endothelial cell migration [51]. All these complexes contain the canonical Nox organizer p47phox, which is cytosolic and targeted to membranes after phosphorylation [154, 155]. In contrast, the NADPH oxidase complexes described at invadopodia seem to contain the Tks proteins as organizers. Tks5 localizes to mature invadopodia and it is recruited to nascent invadopodia by binding of the PX domain to discrete membrane domains enriched in PtdIns(3,4)P2 and PtdIns(3,4,5)P3 [70]. Whether Nox1 or Nox4 are localized to invadopodial membranes before Tks adaptor proteins are recruited to these sites is unknown. The recruitment of Nox1-p22phox or Nox4-p22phox complexes to Tks-enriched membrane domains has been suggested to involve the targeted fusion of Nox-containing vesicles [156]. Indeed, NADPH oxidases are present in internal vesicles [26], and invadopodia are sites of active vesicle trafficking for delivering of transmembrane components as well as exocytosis of proteases [140, 157].

Could NADPH complexes containing other members of the p47phox organizer superfamily (p47phox, p40phox, NoxO1) also be present at invadopodia or at podosomes (the invadopodia counterparts in non-cancer cells)? The PX domain of NoxO1 binds preferentially PtdIns(3,5)P2, PtdIns(5)P and PtdIns(4)P [158], which are not present at invadopodia [70], making it an unlikely candidate for organizing invadopodial or podosomal NADPH complexes. Furthermore, overexpression of NoxO1 in colon cancer cells reduces invadopodia formation, likely by competing with Tks4 to bind NoxA1 [64]. The PX domain of p40phox binds preferentially to PtdIns(3)P but has some affinity for PtdIns(3,4,5)P3 [74, 159], which is also present at invadopodia [70], making it a potential component of invadopodia NADPH complexes. p47phox is another potential candidate, since its PX domain has affinity for PtdIns(3,4)P2 [74, 159], which is enriched at invadopodia [70], and p47phox mRNA has been detected in colon cancer samples [78]. Furthermore, p47phox has been described to bind cortactin [160], IQGAP1 [152], and the paxillin paralogue Hic-5 [51]. Cortactin, IQGAP1 and paxillin are also present at invadopodia [161, 162]. Conversely, one could also speculate that p47phox binding proteins such as WAVE1 [153], moesin [163], coronin [164] or TRAF4 [51] could bind to Tks adaptors at invadopodia or podosomes. ROS are also necessary for podosome formation in normal macrophages [63], indicating that a similar mechanism is at play in controlling invasive cellular microdomains in normal cells and cell invasion in non-diseased tissues.

Inhibition of Nox activity in cancer cells leads to a specific decrease in the levels of tyrosine phosphorylation of Tks5 and Tks4 [63], indicating that redox signaling may regulate invadopodia formation by locally inactivating a tyrosine phosphatase that has Tks proteins as its main targets. The silencing of PTP-PEST in transformed cells increases invadopodia number [63], suggesting that the correct timing of invadopodia turnover requires the inactivation of phosphatases, which is achieved by localization of redox signaling to discrete microdomains. A similar mechanism controls focal adhesion turnover [165]. Correct assembly and disassembly of adhesive structures is necessary for cellular migration [165–167] as is the coordination of migration and pericellular proteolysis for local invasion. Consistent with a function of PTP-PEST in the regulation of invadopodia formation, other phosphatases have been shown to regulate the formation of these structures, including PTP1B [168], synaptojanin2 [148] and PTEN [169] in different cell types. Alternatively,

redox activation of Src could increase Tks5 phosphorylation directly, although that possibility remains to be proven. The proposed model for redox signaling control at invasive microdomains by novel invadopodial NADPH complexes containing Tks proteins in cancer cells is summarized in Figure 3.

Implications for cancer therapy

Redox chemotherapeutics are drugs that target either the antioxidant system of cancer cells (taking advantage of the fact that cancer cells are less tolerant to a decrease in antioxidants than normal cells), or the pro-oxidant system of cancer cells, to allow them to proliferative and survive under oxidative stress. These type of drugs are currently in clinical trials [170]. NADPH oxidases are considered as targets for cancer therapy [22, 171, 172], and several NADPH oxidase inhibitors have been developed [173–175]. Some of them decrease angiogenesis or tumor growth in animal models [133, 176]. The implication of Nox1 and Nox4 in the redox control of invasive cellular structures further supports the use of Nox1 or Nox4 inhibitors as anti-invasive drugs. The levels of transcript expression for Nox1 and/or Nox4 are often higher in tumor samples when compared with normal tissue [78], and both Nox1 and Nox4 deficient mice are viable [177, 178], supporting the possible therapeutic use of Nox1/4 inhibitors in humans. Recent findings in transgenic mice suggest that Nox4 inhibitors could be also useful in the treatment of ischemic stroke and hypertension, although cardiotoxicity may be a limitation [178, 179]. Nevertheless, it is worth testing whether Nox-isoform specific inhibitors would make promising anti-cancer drugs.

CONCLUSIONS

ROS are important mediators of normal and pathological cellular processes by acting as second messengers in the process of redox signaling. Cancer cells have evolved to use redox signaling pathways to drive aberrant proliferation, survival and invasion, and to adapt to the tumor microenvironment. By targeting redox signaling to invasive microdomains, such as invadopodia, cancer cells maintain a pro-invasive program that contributes to tumor cell invasion and metastasis. The invadopodia NADPH oxidase complexes that drive redox signaling at invasive microdomains represent a new mechanism by which redox signaling controls cancer progression and suggest the possibility of targeting invadopodia-specific NADPH oxidase components for therapeutic purposes.

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ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
Cdc25C	cell division cycle 25 homologue C
DCF-DA	5-(and-6)-carboxy-2',7' -dichlorofluorescein
Duox	Dual oxidase
E-Cadherin	epithelial cadherin
EC-SOD	extracellular superoxide dismutase
ECM	extracellular matrix

EGF	epithelial growth factor
EMT	epithelial to mesenchymal transition
FAK	focal adhesion kinase
Grb2	growth factor receptor bound protein 2
Hic-5	hydrogen peroxide-inducible clone 5
HIF1α	hypoxia inducible factor 1 alpha
IQGAP1	IQ motif containing GTPase activating protein 1
JNK	Jun N-terminal kinase
k-Ras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LMW-PTP	low molecular weight protein tyrosine phosphatase
MARCKS	myristoylated alanine-rich protein kinase C substrate
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1 metalloprotease
N-WASP	neuronal Wiskott-Aldrich syndrome protein
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
Nck	non-catalytic region of tyrosine kinase adaptor protein 1
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nox	NADPH oxidase
NoxA1	NADPH oxidase adaptor 1
NoxO1	NADPH oxidase organizer 1
p190RhoGAP	p190 Rho GTPase activating protein
p22phox	p22 phagocytic oxidase
p40phox	p40 phagocytic oxidase
p47phox	p47 phagocytic oxidase
p67phox	p67 phagocytic oxidase
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
Poldip2	polymerase (DNA-directed), delta interacting protein 2
PtdIns(3,4,5)P3	phosphatidylinositol (3,4,5)-trisphosphate
PtdIns(3,4)P2	phosphatidylinositol (3,4)-bisphosphate
PtdIns(3,5)P2	phosphatidylinositol (3,5)-bisphosphate
PtdIns(3)P	phosphatidylinositol 3-phosphate
PtdIns(4)P	phosphatidylinositol 4-phosphate
PtdIns(5)P	phosphatidylinositol 5-phosphate

PTEN	phosphatase and tensin homolog
PTP-PEST	Protein tyrosine phosphatase with a C-terminal PEST motif
PTP1B	protein tyrosine phosphatase 1B
PTP	protein tyrosine phosphatase
PX	phox homology
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SH3	Src homology 3 domain
SOD1	superoxide dismutase 1
Stat3	signal transducer and activator of transcription 3
TGFβ	transforming growth factor beta
Tks4	tyrosine kinase substrate with four SH3 domains
Tks5	tyrosine kinase substrate with five SH3 domains
TPA	12-O-Tetradecanoylphorbol-13-acetate
VEGF	vascular endothelial growth factor
WASP	family verprolin-homologous protein

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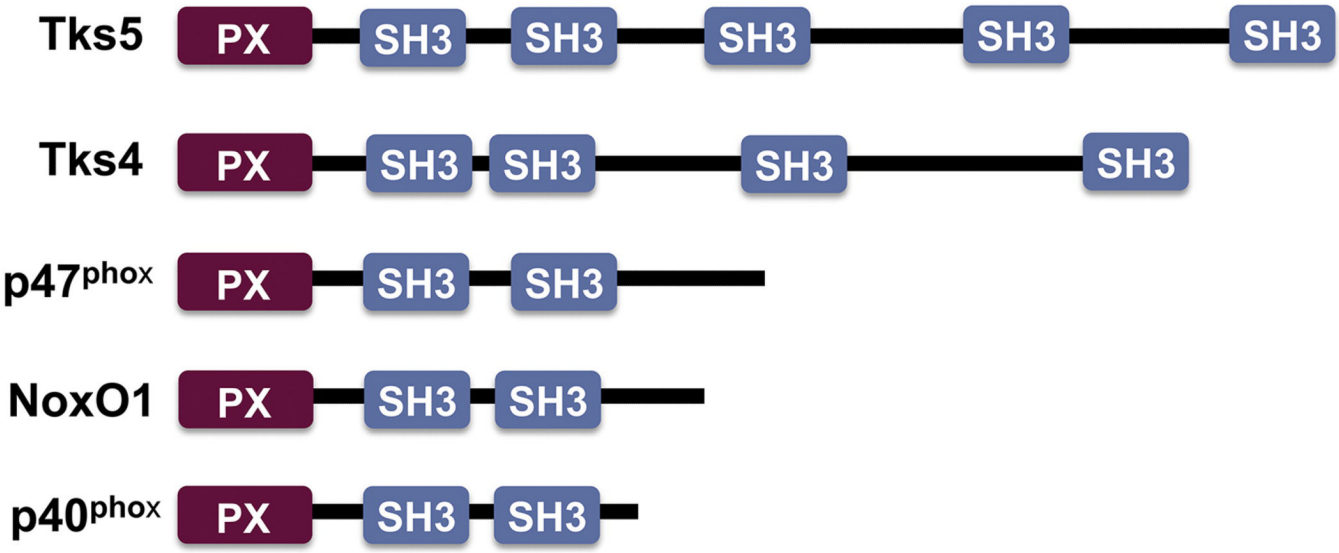

Figure 1.
Comparison between the structural domain organization of Tks proteins and their homologues p47phox, NoxO1 and p40phox.

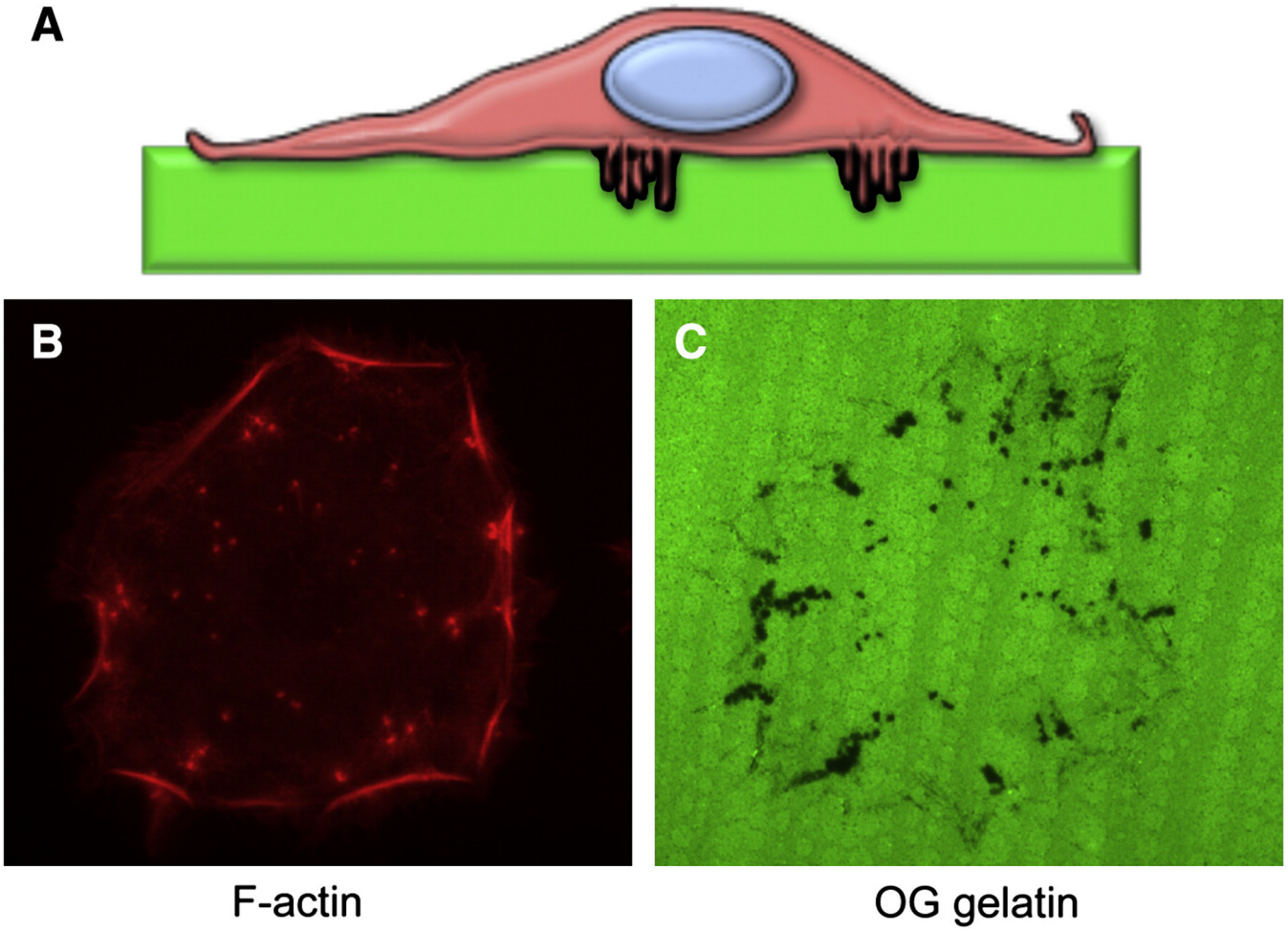

Figure 2. Invadopodia are invasive microdomains

A. Diagram of a cancer cell growing on top of a fluorescent ECM substrate. Invadopodia are thin cellular protrusions from the cell-substrate interface into the substrate.

B. F-actin staining of a head and neck carcinoma cell showing invadopodia (dots).

C. OregonGreen-labeled gelatin from the same field as B) showing the areas of invadopodia degradation (black dots).

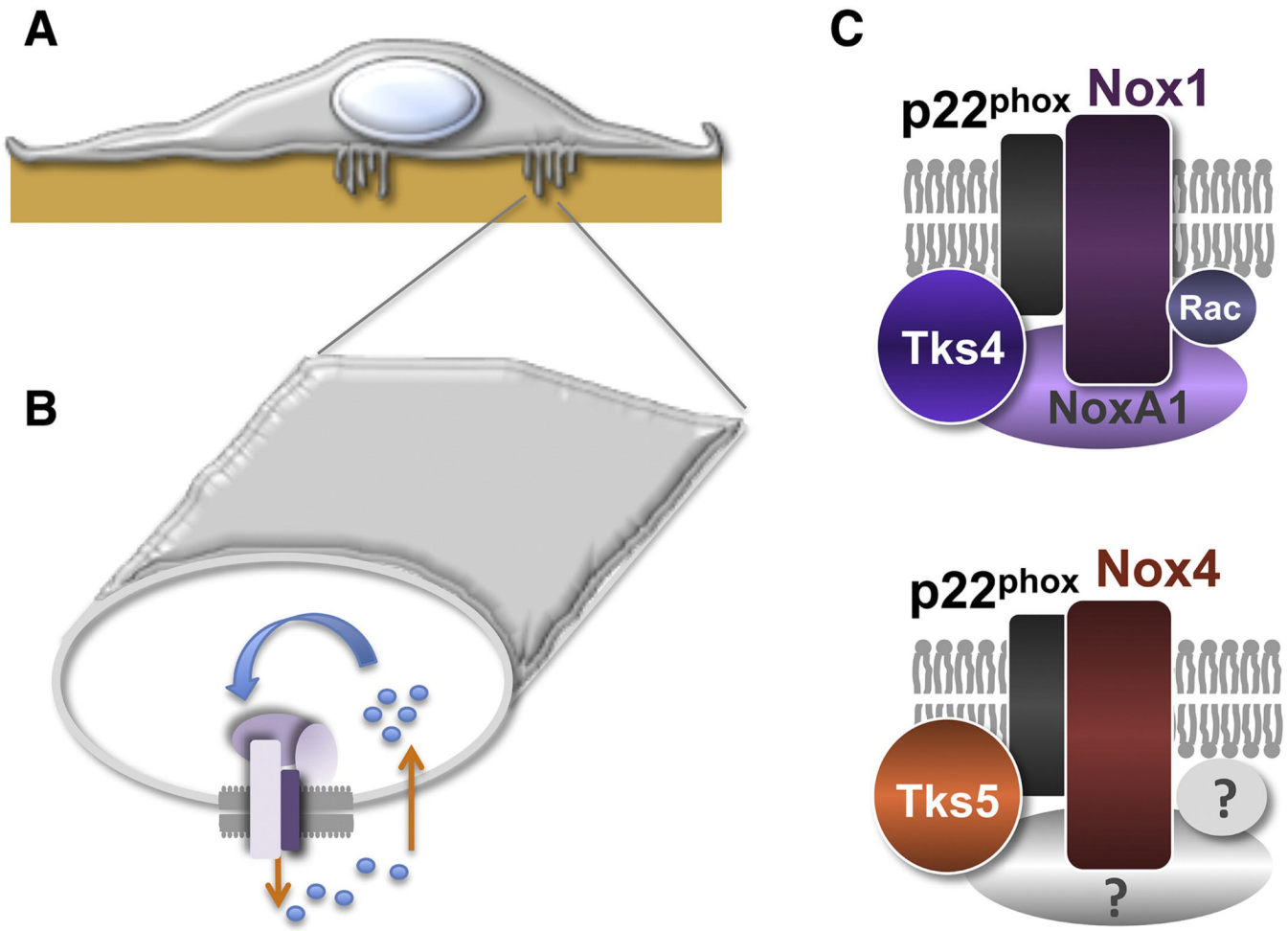

Figure 3. Invadopodia NADPH oxidase complexes regulate redox signaling at invasive microdomains

A. Diagram of a cancer cell with invadopodia extending into the ECM substrate.

B. Diagram showing the section of an invadopodium and the localization of NADPH oxidase complexes that mediate local generation of ROS (dots) to regulate invadopodia formation and activity.

C. Specific NADPH oxidase complexes containing Tks4 and Tks5 proteins might be locally present at invadopodia. Question marks denote possible additional components of the invadopodia complex containing Nox4.

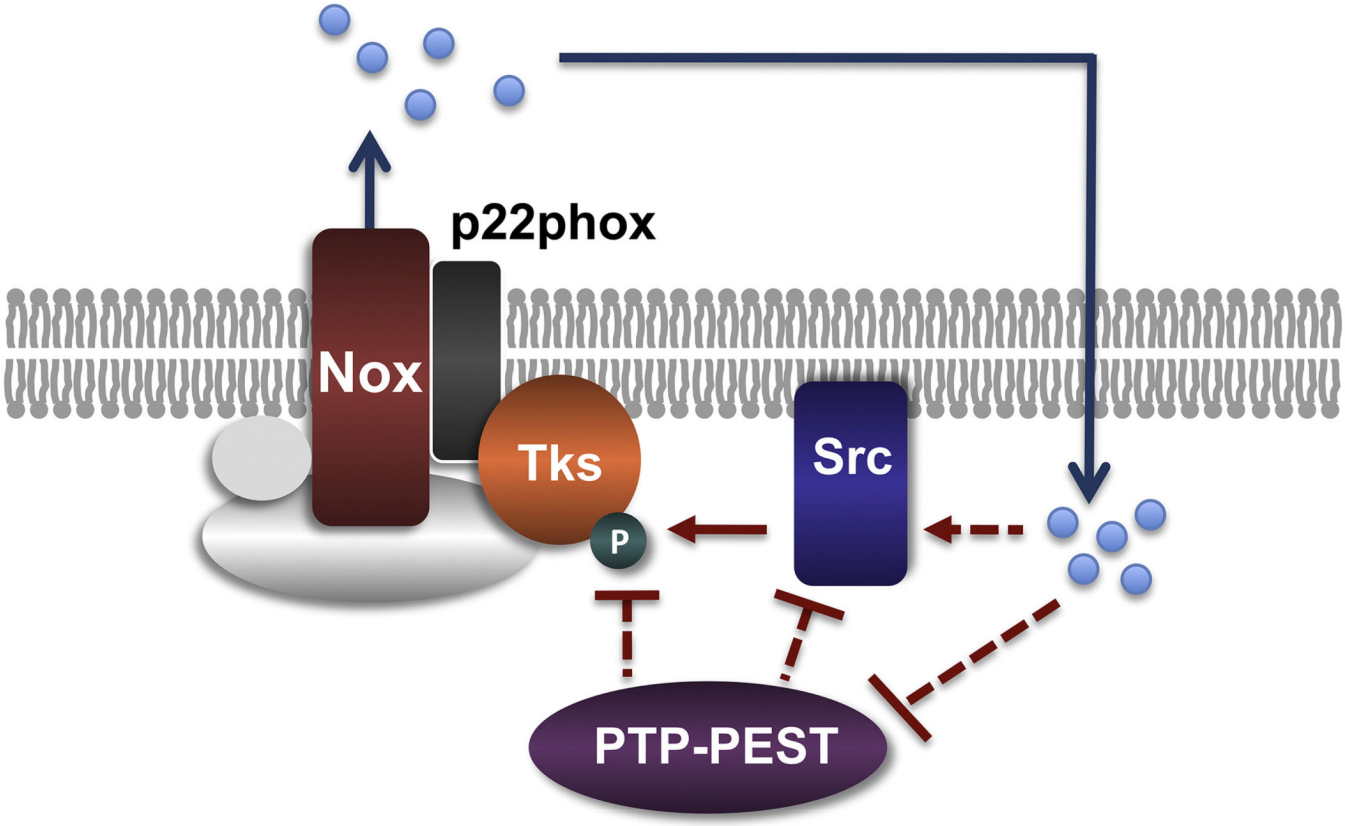

Figure 4. Model for a redox signaling pathway at invadopodia

An active invadopodia NADPH oxidase complex containing a Tks protein family as an organizer generates ROS (dots) that locally amplify the tyrosine-phosphorylation signal initiated by Src-dependent phosphorylation of Tks proteins. The intensity and/or duration of the Tks tyrosine phosphorylation could be modulated by ROS through direct activation of Src or by inactivation of the phosphatase PTP-PEST, which may in turn dephosphorylate Src or Tks organizers. Dashed lines with question marks denote putative interactions.