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The role of NADPH oxidase and ROS in invadopodia formation

Bachelor's thesis

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I declare that I wrote this bachelor's thesis alone on the basis of listed literature and consultations with an advisor.

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Outline

Abstract

Invadopodia as specific organelles enabling tumour cells movement, spreading over the organism and ultimately formation of metastasis are possible and promising targets of tumour therapy. Recently, many interesting facts about assembly and mechanism of function of invadopodia were discovered. Invadopodia are centres of ECM degradation by extra-cellular proteases facilitating an invasion of tumour cells. For creation of invadopodia a precisely localized increased production of ROS is necessary. ROS work as crucial signalling molecules and participate in many processes resulting in invadopodia formation. ROS in tumour cells are produced by specific extra-mitochondrial NADPH oxidases (Nox). Several regulatory molecules participating in activation and localization of Nox to invadopodia have been discovered recently (Tks organizer proteins). Furthermore, a regulatory role of Src kinase in ROS production and subsequent invadopodia formation was confirmed.

Key words: ECM degradation, invadopodia, invasion, proteases, Nox, ROS, Src kinase, Tks proteins

Abstrakt

Invadopodia, jako specifické organely nádorových buněk umožňující jim pohyb a tudíž i šíření po organismu a tvorbu metastáz, jsou jedním z možných slibných cílů pro nádorovou terapii. V poslední době vyšly na jevo mnohé zajímavé skutečnosti o vzniku invadopodií a mechanismu jejich fungování. Invadopodia jsou místy degradace ECM extracelulárními proteázami umožňující invazivitu nádorových buněk. Pro vznik invadopodií je nezbytná přesně lokalizovaná zvýšená tvorba reaktivních kyslíkových radikálů, které se jako důležité signální molekuly účastní mnoha dějů vedoucích k tvorbě invadopodií. ROS v nádorových buňkách jsou produkovány především specifickými extramitochondriálními NADPH oxidázami (Nox). V poslední době bylo odhaleno několik regulačních molekul, které se podílí na vzniku těchto Nox a jejich lokalizaci do místa vzniku invadopodií (Tks organizační proteiny). Dále byla v produkci ROS a následném vzniku invadopodií potvrzena regulační role kinázy Src.

Klíčová slova: degradace ECM, invadopodia, invaze, proteázy, Nox, ROS, Src kináza, Tks proteiny

1. Introduction

Spreading of cancer cells into distant parts of a human body and creation of metastasis is the most frequent cause of death of tumour patients. Invasive tumour cells have special organelles called invadopodia that are centres of ECM degradation, which allows the metastatic cells to pass through the tissue barriers to lymphatic or vascular system and expand all over the organism (Weaver, 2006). The generation of ROS by NADPH oxidases and the formation of invadopodia seem to be the two key features that result in invasive behaviour of cancer cells (Diaz et al., 2009).

The understanding of mechanisms of invadopodia formation and function can be crucial for development of a new anti-invasive therapy. Presence or absence of invadopodia has no effect on a viability of the cells and this fact suggests an idea that therapy targeting at invadopodia might have less negative side effects than methods used now such as chemotherapy or radiotheraphy (Weaver, 2006).

2. Invadopodia

Invadopodia are structures characteristic for invasive cancer cells which are able of proteolytic degradation of ECM. Growing tumours need to remodel the surrounding ECM to make space for new cells (Gimona et al., 2008) but also to invade across basement membranes, grow at secondary sites and create metastasis (Weaver, 2008).

2.1. Invadopodia versus podosomes

Invadopodia and podosomes are transient cellular organelles (sometimes called invadosomes) (Linder, 2009) that seem to have a lot in common but also differ in many aspects. They share some functional and molecular characteristics including proteins such as integrins, proteases, dependence on Src kinase and branched actin assembly (Weaver, 2006), but their relationship, ability to transform into each other or a common precursor have not been proved yet (Linder, 2009).

2.1.1. Invadopodia

Invadopodia are protrusive actine-rich membrane structures of ventral plasma membrane. They are found on invasive cancer cells or Src-transformed cells, they are closely associated with proteolytic ECM degradation and they are thought to be the key structures in cell invasion (Weaver, 2006).

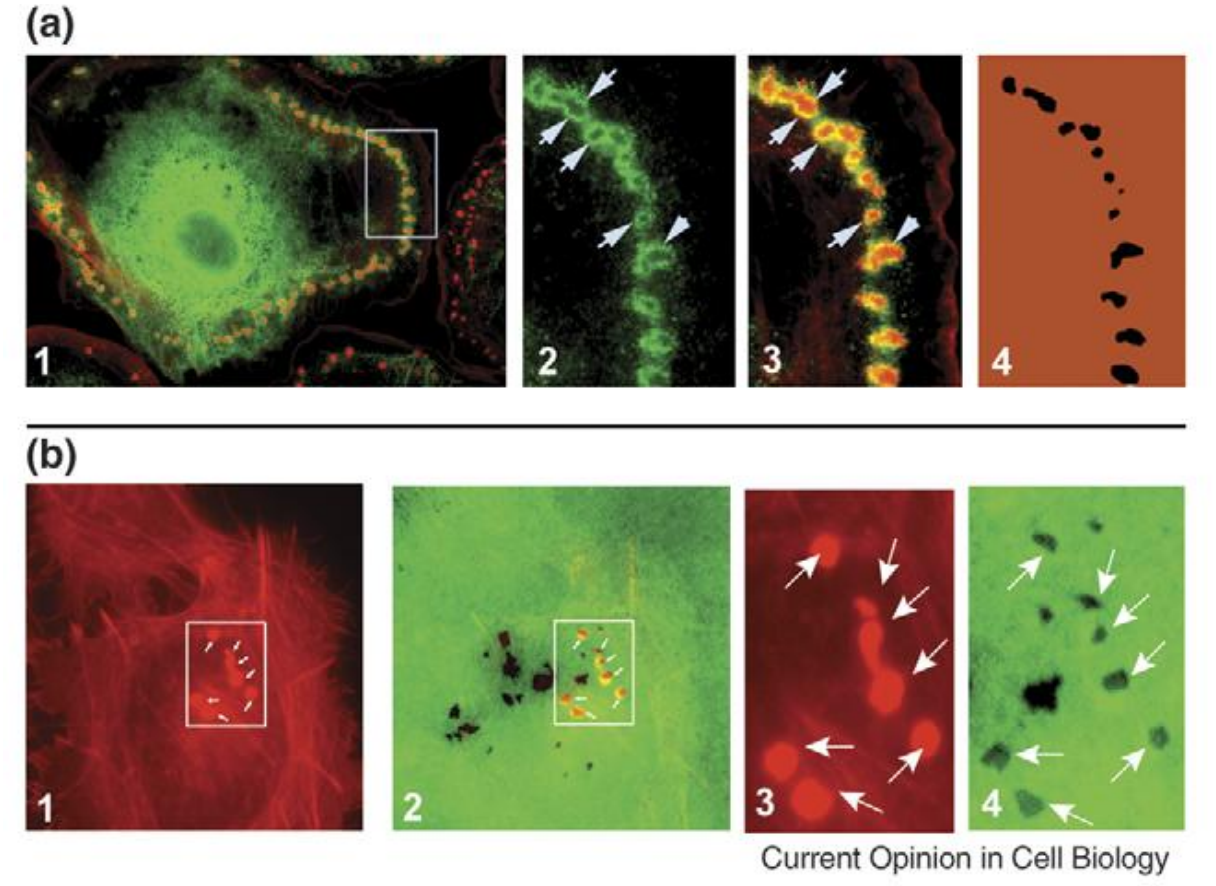
Invadopodia are usually described as punctate finger-like projections extending into ECM, localized in vicinity of cell nucleus and in proximity of Golgi complex (Albiges-Rizo et al., 2009). Their dimension is from 0.5 μm up to 8 μm. They are formed constitutively and number of invadopodia in one cell varies from 1 to 100. They are quite stable with a life-time up to one hour (Linder, 2009).

2.1.2. Podosomes

Podosomes are also actin-rich membrane structures associated with ECM degradation, but they are typically found on invasive non-malignant cells such as macrophages and osteoclasts that remodel tissues so podosomes do actually have a physiological role in bone resorption and necessary ECM degradation (Gimona et al., 2008).

Podosomes form characteristic ring-like structures of adhesive proteins surrounding an actin-rich core. Some of these adhesive proteins are also present in invadopodia, but one of them (vinculin) is podosome-specific and serves as podosome marker (Gimona et al., 2008). Podosomes are formed only after activating signal and they are much smaller than invadopodia, their dimension is 1 μm maximum. Cells usually form more than 100 podosomes with a life-time of several minutes (Linder, 2009).

Figure 1



(a) Podosomes in cultured smooth muscle cells. Ring structures (here marked by the cytolinker protein plectin in green; 2 and 3) surround F-actin-rich cores (in red, 3) that mark sites of ECM degradation (black areas in 4).

(b) Invadopodia in human melanoma cells. F-actin-rich dots (in red, 1 and 3) overlap with areas of ECM (in green, 2 and 4) degradation (black patches in 2 and 4).

Source: **Gimona M, Buccione R, Courtneidge SA, Linder S**. 2008. Assembly and biological role of podosomes and invadopodia, *Curr Opin Cell Biol*, 20(2):235-41.

2.2. Structure of invadopodia

Invadopodia are membrane protrusions formed by polymerized actin filaments that create a branched actin network. They are localized on ventral side of tumour cells. The basal structure of invadopodia is an actin-rich core containing branched actin, actin nucleation machinery (WASP, N-WASP, WIP, Arp2/3 complex) and cortactin, surrounded by an adhesive ring (focal adhesion) that consists of integrins and integrin-associated proteins such as talin or paxillin that connect integrins to actin cytoskeleton (Albiges-Rizo et al., 2009; Paňková et al., 2010).

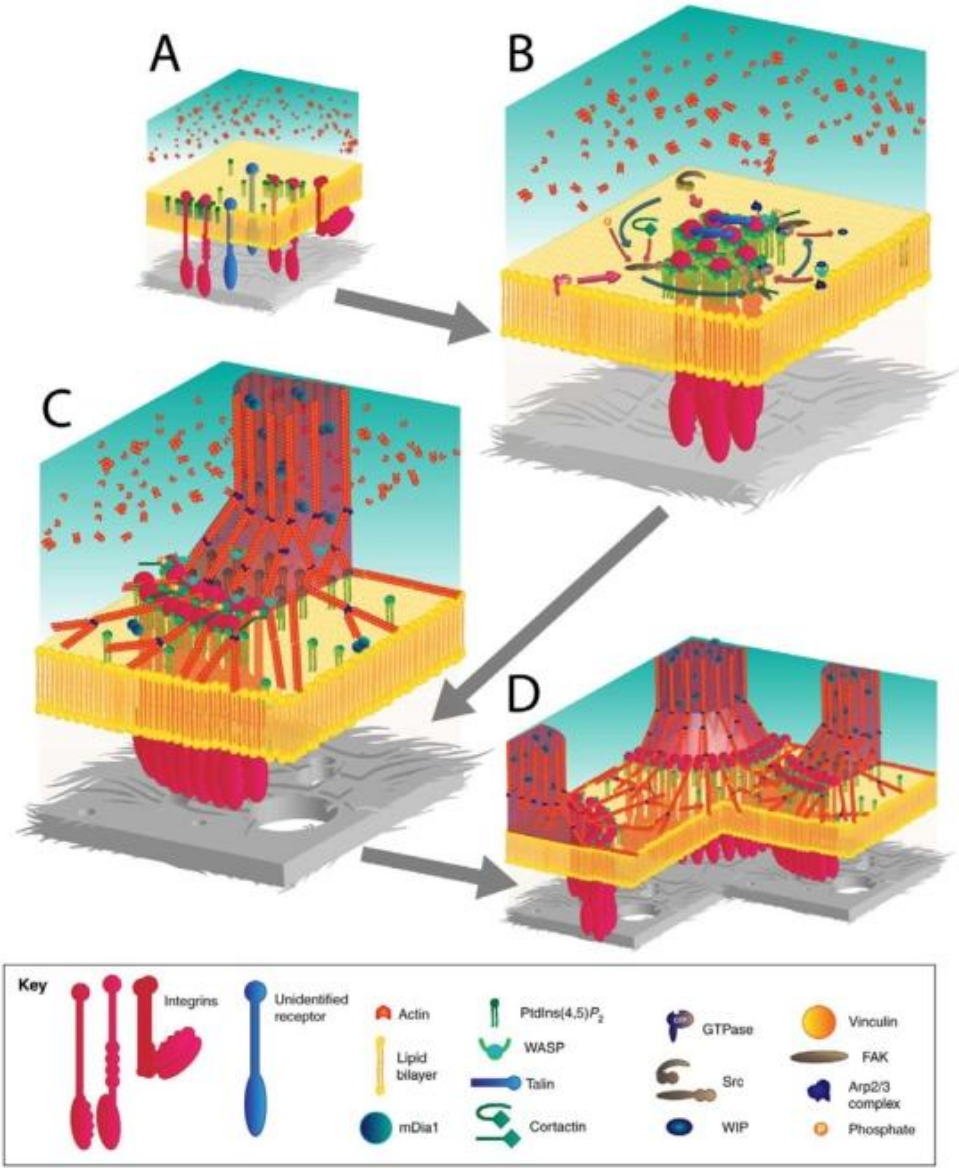
Invadopodia formation begins with nucleation of F-actin and the nascent filaments are oriented perpendicularly to the substrate. The actin nucleation is based on Arp2/3 complex interacting with WASP or N-WASP regulating proteins at plasmatic membrane in presence of WIP. WASP proteins are under control of Rac small GTPase. Cortactin also participates in nucleation and moreover stabilizes the nascent filament branches. Cortactin is a Src substrate and it showed up to be a key molecule for membrane dynamics because its phosphorylation enhances actin assembly (Albiges-Rizo et al., 2009).

Actin nucleation is followed by actin elongation process. The most important elongation factors that conduct actin polymerization are Ena/VASP-family proteins and formins. The protrusive structure contains two F-actin networks with opposite orientations (parallel and perpendicular to substrate). During the life-time of invadopodia a repetitive nucleation, polymerization and fast actin turnover is proceed (Albiges-Rizo et al., 2009).

Besides the actin polymerization machinery, membrane deformation is another activity crucial for invadopodia formation. Membrane curvature requests specific proteins, able to bind and deform plasmatic membrane such as BAR and F-BAR protein families. These proteins deform the membrane in the direction away from cytosol and create tubular protrusive structures up to 80 nm long. Both actin polymerization machinery proteins and membrane deformation proteins are necessary to cooperate in complicated process of protrusive structure formation and their balance decides about its form, quantity and dynamics and if invadopodia do actually form or not (Albiges-Rizo et al., 2009).

The mechanism of movement of invasive cells is mediated by the contractility of actin cytoskeleton regulated by small GTPases from the Rho family, mainly Rac and Cdc42 (Paňková et al., 2010).

Figure 2



Schematic view of signaling pathways that leads to actin organization at invadopodia or podosomes. (A) At the initial stage of adhesion formation, integrins or other unidentified receptors bind to components of the ECM (grey), leading to clustering of receptors into PtdIns(4,5)P_2-enriched areas of plasma membrane. (B) Recruitment of Src to adhesion sites leads to phosphorylation of several proteins such as cortactin, WASP, FAK and regulators of small GTPases. Continuous actin nucleation relies on the continuous and strong activation of the Arp2/3 complex at the membrane through the synergistic action of cortactin and WASP-family proteins. (C) DRF/mDia1 elongates actin filaments into columnar structures from the branched actin network that was previously induced by N-WASP, the Arp2/3 complex and cortactin. (D) Podosomes or invadopodia are mechanically connected through a network of radial actin filaments that lie parallel to the substratum.

Source: Albiges-Rizo C, Destaing O, Fourcade B, Planus E, Block MR. 2009. Actin machinery and mechanosensivity in invadopodia, podosomes and focal adhesions, *J Cell Sci*, 122(pt17): 3037-3049.

2.3. Extra-cellular matrix degradation

ECM digestion is a complex process that requires ensemble of many activities like cytoskeletal assembly, tyrosine kinase signalling, vesicular trafficking and adhesion (Weaver, 2009). The crucial enzymes for ECM degradation are proteases that digest ECM macromolecules such as collagen, fibronectin or lamminin. In various cancer tissues increased levels of ECM-degrading proteinase were found.

Four main groups of proteinases were found to participate in ECM degradation.

2.3.1. Matrix metalloproteinase family (MMPs)

MMPs family includes 25 extra-cellular zinc dependent endopeptidases that are secreted to ECM or anchored to a cellular surface. They consists of a signal domain, pro domain and catalytic domain with three conserved histidines which bind the active site Zn^{2+} (the prefix ‘‘metallo’’). For MMP activation the pro domain has to be disrupted (Chen and Parks, 2009). It has to be mentioned that not all MMPs participate in ECM degradation.

A significant member of MMPs family accumulated to invadopodia is transmembrane MT1-MMP (MMP-14). It is activated by a proteolytic cleavage of pro domain by furin in the trans-Golgi network before it is transported to a plasma membrane (Poincloux et al., 2009). MT1-MMP is a collagenase but besides it ability to digest ECM it has also crucial role as a regulator because it cleaves the pro domain of some secreted forms of MMPs (mostly MMP-2 and MMP-13) and convert them into their active form which leads to more intensive ECM degradation (Binker et al., 2009).

Two other members of MMPs family that participate at ECM degradation and that are frequently present in invadopodia are extra-cellular secreted MMP-2 (gelatinase A) and MMP-9 (gelatinase B). They are able to breakdown the basal membranes by collagen IV digestion (Poincloux et al., 2009), which helps to the cancer cell invasion. For a long time they were considered to be the crucial molecules of ECM degradation but recently MT1-MMP as their regulator acquired key importance.

As mentioned above, not all MMPs are involved in ECM degradation. Several members of MMPs family showed up to have a protective effect in different stages of cancer progression (MMP-8, MMP-12, MMP-26). Transformation of mice with MMP-8 resulted in their anti-tumour protection and knockdown of MMP-8 made mice more cancer predisposed (López-Otín and Matrisian, 2007).

2.3.2. A disintegrine and metalloproteinase domain protease family (ADAMs)

The proteases of ADAMs family are closely related to MMPs family. They share the metalloproteinase domain and they are often over-expressed in invasive cancer cells. Also the activation mechanism of nascent pro-ADAM by furin or MMPs is similar (Mochizuki and Okada, 2007).

ADAMs are divided into two groups: membrane anchored ADAMs (34 members) and ADAMs with thrombospondin motifs ADAMTS (19 members). They participate at ECM degradation specifically in various cancer cell lines (Mochizuki and Okada, 2007), but some of them (ADAM-23, ADAMTS-1,-8,-9,-15-18) also elicit the anti-tumour protective effect analogous to MMPs (López-Otín and Matrisian, 2007).

2.3.3. Urokinase-type plasminogen activator (uPA)

Urokinase-type plasminogen activator (uPA) is a member of a serine protease family with pleiotropic functions. It is secreted in an inactive form pro-uPA that requires its cell surface receptor uPAR for activation. uPA-uPAR complex main function in ECM degradation is conversion of plasminogen into its active form plasmin which is a potent ECM degrading serine protease that cleaves specific proteins at cell-cell and cell-matrix contacts. The level of uPA and uPAR is increased in invasive cells and correlates with advanced disease (Minoo et al., 2010). uPA is able to localize the proteolytic activity on the cell surface which is extremely important for the invasive ability of tumour cells and besides ECM degradation it plays also an important role in cell signalling involved in cell survival and proliferation. Using specific siRNA to down-regulate the expression of uPA and its receptor resulted in considerable loss of cancer cell invasiveness (Subramanian et al., 2006). Besides its regulatory role uPA-uPAR complex also directly participate at ECM degradation by cleaving of fibronectin (Fazioli and Blasi, 1994).

2.3.4. Cathepsins

Cysteine cathepsins belong to a C1 papain family of cysteine proteases. They are mainly intra-cellular proteases but under certain circumstances (in tumour cells) they can be also secreted to ECM. Some of them are secreted as active enzymes but some of them as

pro-enzymes that require for their activation an interaction with surface glykosaminoglycans. Cathepsin B is one of the possible activators of pro-uPA participating at plasminogen cascade but it can also directly digest ECM (Obermajer et al., 2008).

2.3.5. ROS-mediated control of MMPs activity

The process of ECM digestion and possible ways how to block it can be considered in an anti-invasive therapy. MMPs activity is among others under the control of ROS. It was found that stimulation of cells by over-expressed EGF increased activation of Rac1 small GTPase by Src kinase and PI3K which resulted in increased MMP-2 secretion and activation (Binker et al., 2009). Rac1 downstream signalling pathways involve Nox formation and activation of ROS production which might lead to induction of fusion of protease containing vesicles with plasma membrane and their secretion to ECM (Fig4) (Weaver, 2009).

3. The role of ROS in invadopodia formation

The morphological transformation from epithelial cells to motile individually-migrating mesenchymal cells is called epithelial-mesenchymal transition (EMT). A polarized cell forms the leading protrusion which leads to interaction of the leading edge with ECM (Paňková et al., 2010) that is necessary for invadopodia formation because invadopodia exclusively form upon contact with substrate. It was found that number of invadopodia arise when tumour cells are grown on an appropriate substrate such as collagen or gelatin, which suggests that there exists a positive feed-forward mechanism triggering invadopodia formation (Gimona et al., 2008).

The connecting molecules between cytoskeleton and matrix are transmembrane receptors for integrines (such as fibronectin or vitronectin) or a glycoprotein receptor for hyaluronic acid: CD44. Downstream of integrin receptors there is a signalling cascade where Src kinase seems to be a master switch for invadopodia formation. This signalling leads to formation of an actin-rich core by F-actin polymerization which is the basal structure of invadopodia (Linder, 2009). In this process many important regulatory molecules participate and it might represent a point of control, because efficient ECM degradation only occurs when many signals and processes converge (Weaver, 2008).

3.1. NADPH oxidase and ROS

Maintenance of an appropriate level of intra-cellular ROS is important for keeping the redox balance. Mitochondria are the main natural source of ROS in the organism producing ROS as a side product of oxidative phosphorylation-dependent production of adenosin triphosphate. Endoplasmatic reticulum that generates ROS as a consequence of a protein misfolding is another important ROS producer (Diaz et al., 2009).

Last but not least source of ROS is an extra-mitochondrial family of NADPH oxidases called Nox (Nox1-Nox5 and distant Doux1 and Doux2) (Kumar et al., 2008). Nox produce ROS according to formula:

$$NADPH \rightarrow NADP^{+} + H^{+}$$

$$O_2 \rightarrow O_2^{-}$$

$$O_2^{-} + 2H_2O \rightarrow 2H_2O_2$$

ROS such as superoxide O_2^- or peroxide H_2O_2 play an important role as signalling regulators (inhibition of phosphatases, activation of kinases and regulation of ion channels), or bacteria killers, but they are also highly reactive free radicals that cause serious irreversible damages of crucial cellular macromolecules in case their levels increase too much and the organism suffers from an oxidative stress. In cancer cells the level of ROS often correlates with an aggressive phenotype (Kumar et al., 2008).

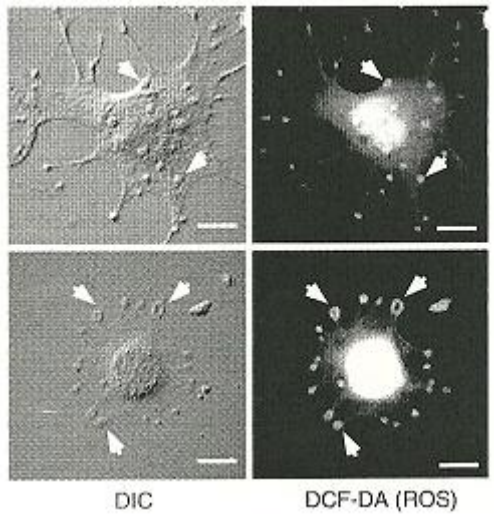
NADPH oxidases were discovered in phagocytes where they participate in a process of oxidative burst which is an important protective mechanism of immune system in case of a pathogen invasion. Later Nox were also found in other tissues and cell types suggesting that they play also other roles (Weaver, 2009). They showed up to participate in many biological processes such as cell growth, apoptosis and angiogenesis. The phagocytic NADPH oxidase is Nox2, the "invadopodial" (especially in Src-transformed cells) are Nox1 and Nox3 and according to latest discoveries also Nox4 (Diaz et al., 2009). An invadopodial specificity of Nox1, Nox3 or Nox4 probably varies with different cell types or cell lines.

The principle of ROS-mediated regulation of redox-sensitive molecules such as kinases or tyrosine, dual-specificity, and lipid phosphatases is based on the ability of ROS (particularly hydrogen peroxide) to catalyze a reversible oxidation (conversion of the sulfhydryl group to sulfenic acid) of cysteine residues in the catalytic site of phosphatase. This leads to phosphatase inactivation and at the same time enhances the kinase activity by removal of an antagonistic phosphatase activity (Weaver, 2009; Gianni et al., 2009; Diaz et al., 2009). An example is ROS-mediated PTP-PEST inactivation resulting in elevation of Src kinase activity and invadopodia formation (Fig4). This was confirmed also by experimental knockdown of PTP-PEST with specific siRNA in Src-transformed cells that increased the number of invadopodia from average 5 up to 20 per cell (Diaz et al., 2009).

Another possible function of ROS in invadopodia formation is direct or indirect activation of MMPs, protein kinase C, Src kinase family and MAPK signalling pathways (Diaz et al., 2009).

The sub-cellular localization of ROS can be determined by incubation of cells with ROS sensor CM-DCF-DA and visualization with differential interference microscopy (DIC) and fluorescence microscopy (DCF-DA). Plenty of ROS are present in invadopodia (Fig3) (Diaz et al., 2009).

Figure 3



Some ROS localize to invadopodia. Src-3T3 cells were incubated with the ROS probe CM-DCF-DA and visualized under DIC and fluorescence (DCF-DA) microscopy. Arrows indicate rosettes of invadopodia. Scale bar 5 μm.

Source: Diaz B, Shani G, Pass I, Anderson D, Quintavalle M, Courtneidge SA. 2009. Tks5-dependent nox mediated generation of reactive oxygen species is necessary for invadopodia formation, *Sci Signal*, 15;2(88):ra53.

3.1.1. Tks4 and Tks5 proteins as Nox organizers

Tks4 and Tks5 are invadopodia-specific scaffold proteins that are essential for invadopodia function because knockdown of either protein blocked invadopodia function and even formation. Tks5 is a Src substrate consisting of N-terminal PX domain (*phox*-homology), five SH3 domains, several polyproline motifs and two Src phosphorylation sites. Tks4 is a close ortholog of Tks5 but it contains just four SH3 domains (Diaz et al., 2009).

A sequence analysis of Tks pointed out to a homology with $p47^{phox}$ protein and put them together into $p47^{phox}$ -related organizers superfamily sharing a common ancestor. Proteins of this family serve as regulatory components for Nox assembly in response to various signals (Gianni et al., 2009). $p47^{phox}$ is a primary regulator of phagocytic Nox2 assembly. In activated phagocytes $p47^{phox}$ (called also NoxO2) is phosphorylated, associated with other cytosolic components ($p67^{phox}$ called also NoxA2 and Rac2 small GTPase) and the whole complex is transported to plasmatic membrane where it joins Nox2-$p22^{phox}$ complex and activates Nox2 (Weaver, 2009). The connection of organizers and $p22^{phox}$ is mediated by SH3 domains (Diaz et al., 2009).

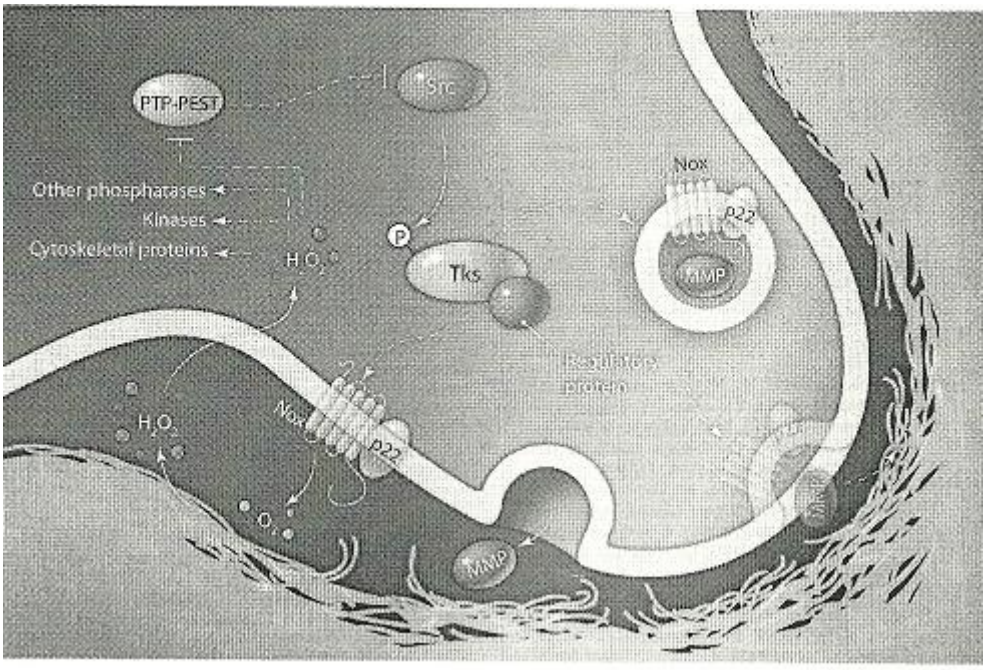
Tks proteins support selectively Nox1 and Nox3 assembly (Gianni et al., 2009) simultaneously with NoxO1 organizer, but only Tks aim Nox to invadopodia (in Src-transformed cells). Over-expression of antagonistic NoxO1 organizer that aims Nox to other cellular compartments resulted in reduction of invadopodia formation (Gianni et al., 2009). For effective invadopodia formation Tks must be present in much higher relative abundance than NoxO1 (Diaz et al., 2009).

Besides Nox organizers other proteins necessary for Nox1 and Nox3 full activation and invadopodia formation are Rac1 small GTPase and NoxA1 activator that binds to Nox organizers (NoxO1, Tks4 and Tks5). NoxA1 binds independently on Rac1 GTP state to one of the organizer's SH3 domains (Gianni et al., 2009). The function of Rac1 and NoxA1 is catalysis of electron transfer from NADPH to FAD with concomitant superoxide production (Diaz et al., 2009).

To summarize Tks showed up to be Nox organizer proteins that have an influence on Nox activity (Weaver, 2009). They affect localized ROS production that is necessary for invadopodia formation (Diaz et al., 2009). ROS in return modulate Tks tyrosine phosphorylation and activation in a positive-feedback loop (Diaz et al., 2009).

Despite of the generally accepted theory that Tks are Nox1 and Nox3 specific, particularly in B16-F10 melanoma cells transfected with Nox4 and $p22^{phox}$ also Tks5 was required. It might differ for certain cell backgrounds (Diaz et al., 2009). Further investigation is needed.

Figure 4



Potential ROS signalling pathways in invadopodia. Phosphorylation of Tks4 or Tks5 may allow recruitment to plasma membrane-localized Nox enzymes at nascent invadopodia sites. Translocation of Nox regulatory proteins associated with Tks proteins (Tks) ledas to the binding of $p22^{phox}$-Nox complexes, activation of Nox activity, and production of reactive oxygen species such as O_2^- and H_2O_2. Oxidation of amino sensitive acids in various invadopodia proteins, such as PTP-PEST, leads to enhanced formation and function of invadopodia. Feedback may occur from PTP-PEST to Src. Fusion of Nox-containing vesicles is also shown, in analogy to the fusion of phagosomes that occurs downstream of Nox2 activation in phagocytes. Although it is speculation at the same time, an exciting possibility that Nox activation could affect fusion of MMP-containing vesicles with the plasma membrane.

Source: Weaver AM. 2009. Regulation of cancer invasion by reactive oxygen species and Tks family scaffold proteins, *Sci Signal*,15;2(88):pe56.

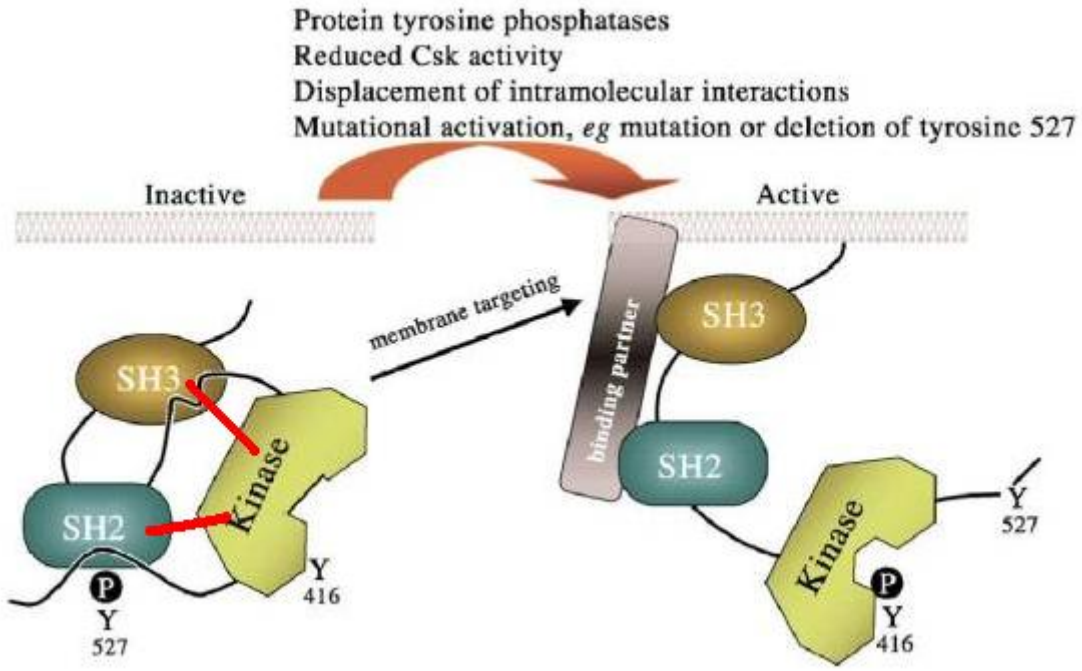
3.1.2. Src kinase

Peyton Rous was the first to declare that cancer might be caused by virus (v-Src containing retrovirus RSV that was called in honour of his discoverer). This theory led to lots of experiments bringing up important facts about cancer and among others Src kinase as a product of *src* gene was discovered. Src was the first tyrosine-specific kinase ever to be identified (Martin, 2001).

Src family includes 9 enzymes with similar structure that are partially redundant in functions within cell. Characteristic features are myristoylated N-terminal domain that aims Src kinase to membrane, two Src-homology-protein-binding domains (SH2 and SH3)

important for protein-protein as well as intra-molecular interactions, and the tyrosine-kinase catalytic domain with an active site. Src exists in inactive "closed" conformation or active "opened" conformation. In tumour cells or Src-transformed cells the Src kinase is deregulated either by mutation or by disruption of intra-molecular interactions. This leads to deregulation of many processes including actin cytoskeleton reorganization which may result in invadopodia formation (Albiges-Rizo et al., 2009).

Figure 5



Inactive form of Src has phosphorylated Tyr527 which binds SH2 domain in a "closed" inactive conformation. Tyr527 state of phosphorylation is a result of various upstream regulators such as CSK kinase (phosphorylation), or SHP-1 phosphorylase (dephosphorylation).

Activation of Src starts with Tyr527 dephosphprylation that leads to Tyr416 autophosphorylation. Src with Tyr416 phosphorylated is in "opened" active conformation that allows contact with substrate.

Source: http://www.ebi.ac.uk/interpro/potm/2003_2/Page_2.htm. Reprinted from Biochim Biophys Acta 1602(2), M. Frame, Src in Cancer, 114-130, 2002.

Note: The red line modifications in the picture indicate the regulatory SH2-kinase and SH3-kinase interactions.

Many types of invasive cancer cells have increased Src kinase activity. Elevated Src kinase activity often correlates with progression of malignant tumours to metastatic stage. Src kinase signalling is also necessary for invadopodia formation. Many invadopodial proteins are Src substrates including cortactin, Tks4 and Tks5 (Gianni et al., 2009) which fortifies the concept of Src importance and its influence on invadopodia function and

formation. Src-induced ROS production is mediated by Tks phosphorylation resulting in Nox organization and invadopodia formation (Weaver, 2009; Diaz et al., 2009).

Src kinase is also involved in a control of cell proliferation, a cell cycle control and regulation of cellular motility and invasiveness through phosphorylation of cytoskeletal components, by regulation of activity of Rho-family small GTPases and by elevation of synthesis and secretion of proteases that degrade the extra-cellular matrix (Martin, 2001).

3.1.3. Restriction of ROS production

ROS are very unstable and they are rapidly inactivated in cells, so instead of their neutralization by antioxidants such as NAC which brings only temporary results, it is better to target at their production. Blocking of ROS production leads to decrease of cell invasion and ECM degradation and even increase of cancer cell death by loss of mitochondrial redox potential (Kumar et al., 2008).

DPI abrogates ROS production by a specific Nox inhibition (Gianni et al., 2009). After DPI treatment of Src-transformed cells, there were still few punctate actin-rich sturctures left but with remarkable lower actin concentration. Moreover the cells ceased to express MT1-MMP that in addition to its own function is necessary for conversion of pro-MMP-2 into its active form MMP-2 so the cells lost the ability to invade (Diaz et al., 2009).

Another way to abolish Nox mediated ROS production is a use of Nox-specific siRNA (Gianni et al., 2009). For catalytic activity of all the members of Nox family (besides Nox5, Doux1 and Doux2), $p22^{phox}$ is necessary. $p22^{phox}$ can be blocked with specific siRNA which leads to Nox inactivation and decrease of ROS production (Diaz et al., 2009).

It was also observed that knockdown of Tks5 using Tks5-specific shRNA-encoding lentiviruses decreases ROS production in extension comparable to all the just mentioned methods (inhibition of Nox by DPI or $p22^{phox}$ siRNA) (Diaz et al., 2009).

3.1.4. Importance of Nox localization for invadopodia formation

The Nox organizers are able to place Nox to desired localization by distinct phosphoinostol PX domain that only binds to specific acidic phospholipids in plasmatic membrane (Gianni et al., 2009). PX domain of Tks that aim Nox to invadopodia binds only to PI(3,4)P2 (Diaz et al., 2009). The precise localization of Nox is crucial for ROS-mediated signalling because of ROS short life-time and limited ability to diffuse.

Nox can be localized to many cellular compartments such as caveolae and lipids rafts, focal adhesions, phagosomes, lamellipodia, nucleus, invadopodia of cancer cells etc. The localization depends on an interaction with various organizers. As it was mentioned already, Tks localize Nox to invadopodia and only invadopodia-localized Tks-mediated ROS production is responsible for invadopodia formation (Gianni et al., 2009).

It was observed that Nox are usually co-localized with cortacitn in invadopodia, forming special Nox-cortactin structures crucial for efficient ECM degradation because ROS produced by Nox regulate the dynamic of actin cytoskeleton which affects the cell motility (Gianni et al., 2009).

3.2. Extra-cellular redox state and its role in tumour invasion

Redox state is a result of a balance of oxidizing and reducing equivalents and it is different inside and outside the cell. Intra-cellular milieu is reducing and extra-cellular space is known to have more oxidized redox state. There are many factors influencing the final redox state such as redox-modulating proteins (Nox or superoxid dismutase), thiol/disulfide couples and reactive oxygen species or reactive nitrogen species (Chaiswing et al., 2008).

The role of intracellular redox state is well known (see 3.1.) but recently it was revealed that also extra-cellular redox state affects invasive behaviour of cancer cells and that extra-cellular space of cancer cells differs from that of normal non malignant cells.

A protein which is specifically able to regulate extra-cellular redox state is EC-SOD that converts superoxide into oxygen and hydrogen peroxide. It was observed that at least in some cells O_2^- supported invasive behaviour whereas NO inhibited invasiveness. Increasing of O_2^- extra-cellular concentration correlated with increasing MT1-MMP and MMP-2 activity and ECM degradation, while higher level of NO (that is freely diffusible between intra and extra-cellular space) negatively regulated MT1-MMP and MMP-2 mRNA and expression levels resulting in lower invasiveness (Chaiswing et al., 2008).

4. Conclusion

Invadopodia are crucial for mesenchymal mode of cancer cell invasion. They are found exclusively on invasive tumour cells. In invadopodia, or in their close extra-cellular neighbourhood specific proteases able to digest ECM are localized. ECM degradation is necessary for tumour growth and its invasion to surrounding tissues or distant parts of organism with a use of lymphatic or vascular system for transport.

Invadopodia formation is induced by increased production of ROS by Nox localized specifically to invadopodia (Nox1, Nox3 and eventually Nox4). The important regulatory molecules for Nox activation and distinct localization are Tks organizer proteins. Another crucial molecule is Src kinase that among others regulates Tks proteins. Invasive behaviour of tumour cells is also affected by an extra-cellular redox state.

The importance of ROS as regulators of many processes in cancer cells and the role of invadopodia as structures crucial for cancer cell invasion, were known already for a long time. Recent studies interconnected these two phenomena which brought many new facts as well as questions that need further investigation. The novel approach may potentially result in the development of new anti-invasive drugs aimed directly at invadopodia-specific Nox as a crucial factor required for an invasive behaviour of mesenchymal cancer cells.

A list of used abbreviations

ADAM	a disintegrine and metalloproteinase domain protease
ADAMTS	ADAM with thrombospondin motifs
Arp2/3 complex	complex of 7 actin-like subunits that induce branched actin polymeration
BAR	protein with bin-amphiphysin-rvs domain-conserved protein dimerization domain
Cdc	cyclin dependent kinase
CM-DCF-DA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate,acetyl ester
DPI	diphenyleneiodonium
ECM	extra-cellular matrix
EC-SOD	extra-cellular superoxid dismutase
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
Ena/VASP	enabled/vasodilator-stimulated phosphoprotein
FAD	flacin adenin dinucleotide
MMP	matrix metalloproteinase
MT1-MMP	transmembrane metalloproteinase activating MMP-2
NAC	N-acetyl-L-cystein
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
Nox	reduced nicotinamide adenine dinucleotide phosphate-oxidase
N-WASP	neural Wiskott-Aldrich syndrome protein (found widely spread in different cell types)
PI(3,4)P2	phosphatidylinositol 3,4-biphosphate
PI3K	phosphatidylinositol 3-kinase
PTP-PEST	protein tyrosine phosphatase PEST
PX domain	N-terminal *phox* domain
Rac, Rho	small GTPases
ROS	reactive oxygen species
RSV	Rouse Sarcoma Virus
siRNA	small interfering RNA

Tks4	tyrosine kinase substrate with four SH3 domains
Tks5	tyrosine kinase substrate with five SH3 domains
U-2 OS	human osterogenic sarcoma cells
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
WASP	Wiskott-Aldrich syndrome protein (found in leucocytes)
WIP	WASP interacting protein

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