Chapter 3

Manipulating Redox Signaling to Block Tumor Angiogenesis

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1. Introduction

A tumor consists of a population of rapidly dividing and growing cancer cells. Cancer cells have lost their ability to divide in a controlled fashion and as a consequence they rapidly accumulate mutations. In such way cancer cells (or sub-populations of cancer cells within a tumor) will acquire stronger proliferative capacity [1]. Tumors cannot grow beyond a certain size due to a lack of oxygen and other essential nutrients. Tumors cells have then acquired a specific feature that is to induce blood vessel growth, a process called tumor angiogenesis. Tumor angiogenesis is a necessary and required step for transition from a small harmless cluster of cells to a large tumor [2]. The early induction of tumor vasculature is termed "angiogenic switch", that occurs when a tumor mass reaches about dimensions of 2 mm^2 and moves towards progression. The "angiogenic switch" is a rate-limiting step for tumor growth that is not limited at earliest stages, but occurs also at different stages of tumor-progression. The angiogenic switch induces angiogenic sprouting and new vessels formation and maturation. Activation of angiogenesis in premalignant lesions and dormant metastasis is mandatory for tumor survival. The fact that tumor mass is depending on angiogenesis has driven the medical research towards the characterization of molecular pathways and cellular dynamics for the induction and regulation of angiogenesis.

Tumor angiogenesis is regulated by several growth factors (EGF, TGFα, bFGF, VEGF). Induction of these angiogenic factors is triggered by various stresses [3]. For instance, tissue hypoxia exerts its pro-angiogenic action through various angiogenic factors, the most notable is VEGF (vascular endothelial growth factor), which has been mainly associated with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells [4]. Recently, reactive oxygen species (ROS) have been found to stimulate angiogenic response in the normal and pathological angiogenesis. ROS can cause tissue injury in one hand and

promote tissue repair in another hand by promoting angiogenesis. It thus appears that after causing injury to the cells, ROS promptly initiate the tissue repair process by triggering angiogenic response. Recently, it has been reported that redox signaling may influence pathological angiogenesis as well [5,6].

2. Redox signaling in normal and pathological angiogenesis

Redox signaling is a biochemical communication by free radicals, reactive oxygen species (ROS), and other electronically activated species such as nitric oxide and other oxides of nitrogen acting as biological messengers [7]. Pro- and anti-oxidative species act as second messengers. Pro-oxidative species are physiologically produced by cells and tightly regulated with antioxidant systems. Down-regulation of antioxidant system or up-regulation in production of pro-oxidative species leads to oxidative stress state. This condition is reported as dangerous for cells since it conveys macromolecules damage. Importantly, it has been reported that oxidative stress plays a key role in the regulation of tumor angiogenesis [8]. The complex molecular network that regulates endothelial cells homeostasis during angiogenesis includes molecules sensitive to redox state of biological environment. The redox state is determined by the relative abundance of highly chemically reactive species derived from oxygen (ROS: Reactive Oxygen Species) or nitrogen (RNS: Reactive Nitrogen Species) (Table 1).

ReactiveOxygen Species (ROS)	Symbol	ReactiveNitrogen Species (RNS)	Symbol
Hydroxyl	$OH^{\cdot}$	Nitrous oxide	$NO^{\cdot}$
Superoxyde	$O_2^{\cdot -}$	Peroxynitrate	$OONO^-$
Nitric Oxide	$NO^{\cdot}$	Peroxynitrous acid	ONOOH
Peroxyl	$RO_2^{\cdot}$	Nitroxyl anion	NO^-
Lipid peroxyl	$LOO^{\cdot}$	Nitrogen dioxide	$NO^{\cdot}_2$
Peroxynitrate	$ONOO^-$	Dinitrogen trioxide	N_2O_3
Hydrogen Peroxide	H_2O_2	Nitrous acid	HNO_2
Singlet Oxygen	$^{-1}O_2$	Nitryl chloride	NO_2Cl
Hypochloric acid	HOCl	Nitrosyl cation	NO^+

Table 1. List of oxygen (ROS) and nitrogen (RNS) reactive species commonly found in normal and pathological tissues.

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Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important in regulation of cell survival. In general, moderate levels of ROS/RNS functions as signals to promote cell proliferation and survival, whereas severe increase of ROS/RNS can induce cell death. Under physiologic conditions, the balance between generation and elimination of ROS/RNS maintains the proper function of redox-sensitive signaling proteins. Normally, the redox homeostasis ensures that cells respond properly to endogenous and exogenous stimuli. However, when the redox homeostasis is disturbed, oxidative stress may lead to aberrant cell death and contribute to disease development [9].

Reactive species are highly reactive chemical molecules or ions, characterized by unpaired electrons that react with other molecules in order to stabilize their electron configuration and gain a more stable state. Consequently, the reaction of ROS/RNS with cellular molecules is a damaging reaction of oxidation. Oxidized molecules are dysfunctional and may induce cell death. Initially, the presence of ROS/RNS was linked only to cellular damage and cell degenerative processes. However, accumulating evidences derived from the characterization of mechanisms for buffering and regulating reactive species opened the possibility that oxidative species are important for cellular homeostasis. Reactive species had been also described as second messenger molecules and their interaction with molecules is identified as a post-translational modification (i.e. S- nitrosylation of proteins) that can trigger a specific intracellular signal. At the present, the evidence is that a tight regulation of pro-oxidative species levels is essential for cellular homeostasis and that such regulatory mechanism is fundamental to maintain a safe redox state and activate related redox signaling pathways [10].

In vascular beds, the redox state is mainly modulated by oxygen concentration and by mechanical forces (i.e. shear stress caused by blood flow) [11]. In normal conditions oxygen levels are constant and essential to guarantee sufficient provision for tissues oxygenation. Mechanisms for sensing oxygen tension are based on redox-mediated signaling. During normoxic conditions the transcription factor HIF1α (hypoxia inducible factor) is degraded in a ROS-dependent manner, while during hypoxia the concentration of oxygen is lower and ROS levels are differentially modulated. Consequently, HIF1α couples with HIF1β and activates transcription of genes involved in angiogenesis, vascular remodeling and cell proliferation [12].

Redox signaling events are also activated in endothelial cells during normal angiogenesis for sensing mechanical forces. Shear forces are constantly present on endothelial cells where regulate cell proliferation, survival and migration. Vascular forces exercise a mechanical stimulus that is perceived by endothelial cells and translated into intracellular molecular pathways. Therefore, concomitant to shear forces there is an upregulation in production of RNS and ROS. In adult ECs, the mechanical oscillatory shear stress induces the activation of specific antioxidant enzymes or proteins like peroxiredoxins (Prx) that act as "mechano-sensitive antioxidants" [13]. Moreover, specific antioxidant and protective genes are induced. Shear stress causes upregulation of specific "antioxidant transcriptional factors" Nrf2 and ATF in developing embryonic vasculature as well as in adult ECs [14]. Most of the molecules with oxidative properties that modulate endothelial cell homeostasis in normal conditions are included in redox molecular pathways that are altered in pathogenic angiogenesis [15]. There

are specific oxidized products or redox sensitive proteins that behave differentially. ROS-activated factors play different role in context of pathologic angiogenesis or normal angiogenesis. The ATM kinase protein, which is involved in regulation of endothelial cells survival and proliferation is activated in tumor condition under upregulation of ROS and promotes new vessel formation, while it is not activated in normal vasculature [16]. Oxidative stress triggered by inflammation in tumor conditions (i.e. human melanoma) causes lipid peroxidation with consequent accumulation of an oxidized compound: ω-(2-carboxyethyl)-pyrrole (CEP). The CEP acts as a ligand for Toll-like receptor 2 (TLR2) and induces angiogenesis independently from VEGF [17]. Similarly, oxidized lipid (carboxyalkyl pyrroles, CAPs) molecules bind to their TLRs receptors and activate angiogenesis in some specific pathological conditions such us age related macular degeneration [18].

In the following three different paragraphs we will define the cellular systems regulating redox signaling and how they control molecules and factors clearly involved in angiogenesis. In addition, here we plan to present paragraphs about main sources for production of oxidative species and systems for counteract their products and maintenance of an equilibrated cell redox state. Finally, we will describe molecules sensitive to redox signaling that are known for being part of established pathway for tumor angiogenesis signaling.

3. Molecules generating oxidative species in endothelial cells

In endothelial cells the endogenous production of pro-oxidative species is mainly generated by four different enzymes: NADPH oxidases (NOX), Cyclooxygenases (COX), Xanthine oxidoreductase (XOR), and dysfunctional endothelial NOS (eNOS).

NADPH oxidases (NOX). NADPH oxidases are a family of enzymes composed by seven members: NOX1, NOX2, NOX3, NOX4, NOX5 and two homologues DUOX1, DUOX2. All of them are transmembrane proteins containing a NADPH-binding site, a FAD binding region, heme-binding sites and several subunits that function in the regulation and maturation of enzymes. $p22^{phox}$, DUOX activator 1 (DUAX1) and DUOX activator 2 (DUOX2) are important factors for NOX and DUOX maturation. Among factors important for NOX and DUOX enzyme activation we found $p67^{phox}$, NOX activator1 (NOXA1), small GTPase (RAC1 and RAC2). On the contrary specific regulator of NOX4 is polymerase δ-interacting protein 2 (POLDIP2) and Ca^{2+} ions are specific activators of NOX5 and DUOX1/DUOX2 isoforms. These enzymes are also associated to spatial regulator subunits $p40^{phox}$, $p47^{phox}$ and NOX organizer1 (NOXO1) that are important for the enzyme complex structure [19]. NADPH oxidase catalytic activity consists in the generation of superoxide anions (O_2^-) through an electrons transfer cycle from an electron donor (NADPH) to FAD subunit, heme groups and to a final electron acceptor that is a molecule of oxygen. Activation of NOX4, DUOX1 and DUOX2 results mainly in the release of hydrogen peroxide instead of superoxide anions. The specific role of this enzyme family consists in the production and release of pro-oxidative species. Such class of enzymes is considered one of the main player in the redox signaling in cardiovascular system [20]. NADPH oxidases are expressed in various types of cells along the vascular wall, including

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vascular smooth muscle cells, monocytes and macrophages. NOX1, NOX2, NOX4 and NOX5 are constitutively expressed in the endothelial cells and their functionality is regulated by several vascular conditions like shear stress, hypoxia or stimuli as hormones, cytokines, pro-angiogenic factors [21]. It has been demonstrated that NADPH oxidase are sensitive to pro-angiogenic vascular endothelial growth factor (VEGF) activation and it seems probable that reactive oxygen species derived from their oxidase activity may sustain activated VEGFR2 and promote endothelial cells migration and proliferation [22]. In endothelium the regulation of NOX activity is tightly associated with redox balance, since it has been demonstrated that NOX are involved with a series of cardiovascular disease like hypertension, atherosclerosis or ischemia/reperfusion injury [23]. A specific role of NADPH oxidase activity is also reported during the angiogenesis process [24]. NOX1 activity mediates the interaction between leukocyte cells and endothelium and is involved in the initiation of cell migration. NOX1 levels are sensitive to oscillatory shear stress conditions and are positively regulated by HIF-1 and PDGF. Also NOX1 is involved in angiogenic switch by sustaining VEGF signaling and upregulation of matrix metalloproteinase production [25]. NOX2 is also sensitive to vascular pro-angiogenic factors and is reported to be involved in the regulation of ROS signaling for cytoskeleton organization in ECs migration [26]. NOX4 is the most abundant isoform in endothelial tissues and is responsible for basal superoxide production. As a matter of fact the role of NOX4 in vascular tissues is still far from been understood [27]. NOX5 isoform is present in mammalian cells, but its function can be substituted by other isoforms (i.e. DUOX in rodents). In vitro studies report upregulation of NOX5 stimulates endothelial cells proliferation and organization in microvascular tubules. Also NOX5 is sensitive to pro-angiogenic stimuli like angiopoietins [28]. Considering vascular pro-angiogenic factors tightly regulate by NADPH oxidases several inhibitors have been developed as possible approach to modulate redox signaling in tumor angiogenesis. The most studied NADPH oxidase inhibitors are apocyanin and diphenyleneiodonium (DPI). They are quite non-specific inhibitors since they block assembly of enzyme or electron flow. Emerging new inhibitors for endothelial NOX isoforms are triazolopyrimidines inhibitors such as VAS2870 and VAS3947, whose preliminary in vitro and in vivo studies have been reported beneficial for endothelium dysfunctions under oxidative stress [29].

Cyclooxygenases (COX). Cyclooxygenases act in the rate-limiting step of prostanoids biosynthesis. There are two kinds of enzymes: cyclooxygenase-1 (COX-1) and cyclooxygenase-2, also known as prostaglandin endoperoxide H synthase -1 and -2 (PGHS-1 and PGHS-2). Recently, a cyclooxygenase-1 isoenzyme was identified as COX-3 [30]. Prostanoids are lipid molecules that are produced from all animal cells in response to specific stimuli, like hormones. After activation COX produce prostanoids from free fatty acids, typically from arachidonic acid (AA). In particular COX catalyze the bis-oxygenation of AA into the prostaglandin endoperoxide $PGG_{2,}$ an intermediate molecule that is subsequently converted into different kinds of prostanoids by specific enzymes that are downstream COX. There are five main categories of prostanoid molecules and their specific receptors: 1) prostaglandin D_2 (PGD_2) whose receptors are named DP1-DP-2; 2) prostaglandin E_2 (PGE_2) whose receptors are named EP1-EP4, 3) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) whose receptors are named FP, 4) prostaglandin I_2

(PGI_2) whose receptors are named IP and 5) thromboxane A_2 (TXA_2) whose receptors are named TP. It is also been reported that some categories of prostanoids can bind peroxisome proliferator-activated receptors (PPARs). PPARs are key activators of prostanoids signaling, the binding to their specific G-protein linked receptor activates an intracellular second messenger (i.e. IP_3/cAMP/DAG/Ca^{2+}) that starts a molecular pathway, which is characteristic for each kind of ligand-receptor. Prostanoids are a class of short-life molecules: immediately after their production they are released outside from cells by specific receptors (prostaglandin transporters, PTG), allowing them to act in a paracrine or autocrine way. Prostanoids are implicated in the regulation of several physiological states (i.e. renal system, kidney functions) such us pathological states (i.e. inflammation, cancer). In the cardiovascular system this class of molecules is relevant for the homeostasis of the vasculature. Prostanoids differentially modulate vascular remodeling by direct action on endothelial cells and their progenitors (endothelial progenitor cells, EPCs) as well as on platelets and smooth muscle cells. Mainly prostacyclin PGI_2 and thromboxane (TXA_2) are involved in the regulation of cardiovascular system homeostasis, even though they act in a different way. PGI_2 is synthetized from COX-2 and is a local vasodilator. It also regulates vascular relation by modulation of smooth muscle cells. Moreover PGI_2 limits the aggregation of platelets and favorites angiogenesis by exerting a direct effect on cellular pathways of EPCs [31,32]. There are contradictory studies on PGI_2 action during tumor angiogenesis, it is reported PGI_2 induces tumor angiogenesis by binding to peroxisomes proliferator-activated receptor – δ (PPAR- δ)[33], on the other hand it is also been reported that healthy tissues have higher levels of PGI synthases than tumor cells. So, it has been speculated that tumor cells might induce PGI_2 in neighboring endothelial cells and so they take advantage of its angiogenic property for growth. On the contrary, TXA_2 is synthetized from COX-1 in platelets and promotes vasoconstriction and platelets aggregates. TXA_2 plays an important role in tissue repair as well as on pathological conditions by favouring atherogenesis. Consequently, the ratio between TXA_2 and PGI_2 is fundamental for the maintenance of physiological homeostasis [34]. In tumor conditions there are also prostanoids PGE_2 and $PGF_{2\alpha}$, PGD_2. Signaling of PGE_2 and its receptor is involved in tumor angiogenesis. PGE_2 induces upregulation of metallopeptidase 9 and activates the fibroblast factor receptor type 1 (FGFR1) [35]. Also $PGF_{2\alpha}$ is considered a prostanoids molecule that sustains tumor angiogenesis by inducing activation of EGR-1, HIF-1a and VEGF. Regarding PGD_2 and its receptor DP1 there are evidences for their signaling implication in vessels homeostasis, but there are opposing reports about the role of PGD_2 for normal and tumor angiogenesis [36]. While molecular pathways regarding prostanoids signaling in the regulation of vessels proliferation are not fully described, their metabolism is considered of significant importance for development of anti-angiogenic drugs. Drugs for modulation of prostanoids levels are divided into two classes of molecules: 1) inhibitors of prostanoids biosynthetic enzymes (e.g. limiting prostanoids biosynthesis) and 2) antagonists of prostanoids receptors (e.g. blocking prostanoids 'cellular signaling). The most important drugs of the first class molecules are COX inhibitors like NSAIDs (aspirin, non-selective COX inhibitor) and COXIBs (selective COX-2 inhibitor) whose evidence as chemo preventive agents is yet reported in preclinical studies. Among inhibitor molecules, there are also available inhibitors of terminal prostaglandin

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synthetize (tPGSs), and in particular for mPGES-1 (microsomal prostaglandin E synthetize -1). Inhibitors of mPGES-1, like AF3442e, are now at the beginning of clinical trials. Regarding the second class of drugs, there are many selective and isoform specific molecules. In particular EP antagonists have been successfully tested for limiting angiogenesis in different kinds of pathologies: the ONO8711 (EP1 antagonist) has been tested for inhibitory effects on metastasis and invasion in hepatocellular carcinoma, the EP3 antagonist ONOA23240 has been tested for limiting metastasis in Lewis lung carcinoma, the EP4 antagonists ONOA23208 and AH23848 have been tested for limiting angiogenesis and metastasis in skin melanoma, colorectal adenomas, lung carcinoma and ovarian carcinoma. The limits in application of EP antagonists is the high level of specificity of action: EP isoform antagonists effects are mediated by signaling related to a specific EP isoform relative expression in a tumor, that is always tissue and tumor dependent [37]. An alternative approach that is still needs to be validated is the application of drugs that modulate the activation of PPAR. Even though prostanoids are a heterogeneous class of molecules whose metabolism and signaling still needs to be largely characterized in tumors, they are involved in angiogenesis processes and targetable from drugs.

Xanthine oxidoreductase (XOR). Xanthine oxidoreductase is molybdenum-iron-sulfur flavin hydrolase and, consequently, its essential cofactors are molybdopterin (Mo-Co), two iron-sulfur centers (Fe_2-S_2) and flavin adenine dinucleotide (FAD). XOR shifts between two inter-convertible forms: Xanthine oxidase (XO; EC 1.1.3.22) and Xanthine Dehydrogenase (XDH; EC 1.17.14). XOR enzyme works in the purine degradation pathway where it converts hypoxanthine and xanthine to uric acid. The catalytic reaction consists of an electron flow from precursor molecules to electron acceptors (cofactors). In the first part of reaction xanthine reduces XOR at the Mo-Co core and subsequently the Fe_2-S_2 coordination core mediates the re-oxidation of XOR by reducing FAD into $FADH_2$. In order to restore FAD+, electrons are shifted to NAD+ and in turns directly to oxygen. Consequently, the re-oxidation reaction of XOR yields to two molecules of hydrogen peroxide (H_2O_2) and two molecules of superoxide anion (O_2^-) [38]. XOR enzyme is expressed at highest levels in the gut and in the liver. However, it has been also detected in the heart and in endothelial cells [39]. Behind purine metabolism, XOR is reported to be important for redox signaling since superoxide radicals generated as side products have shown in pathological conditions of cardiovascular system. In vitro overexpression of XOR in cultured endothelial cells reduces cell viability, proliferation and ability to generate vascular tubes due to upregulation of ROS levels [40]. It has also been described that XOR-generated ROS affect heart cardiac contractility by reacting with nitric oxide and generation of $ONOO^-$ species [41] or by regulating myofilaments sensitivity to Ca^{2+} [42]. Moreover XOR activity seems to play a role in oxidative state of infusion /reperfusion injury as well as in myocardial infarction. Treatments with inhibitors of XOR as allopurinol and oxypurinol are reported as benefic for cardiovascular pathologies related to XOR-generated ROS overload. Clinical studies have demonstrated that XOR-inhibition diminishes endothelium dysfunctions by limiting oxidation of molecules and in particular of lipids by favoring vaso-relaxation. In particular allopurinol treatment improves endothelium functions in patients with congestive heart failure by reducing plasma levels of malondyaldehyde (i.e. lipid peroxidation) and improving NO bioavailability [43].

Endothelial Nitric Oxide Synthase (eNOS). eNOS is one of the three isoform of nitric oxide synthase family and it is constitutively expressed in endothelial cells. eNOS is important since it is the major source of endothelial nitric oxide (NO). NOS enzymes work as homodimers with support of several cofactors. One monomer is linked to flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and binds the second monomer at the oxygenase domain that contains a prosthetic heme group. The second monomer is also linked to cofactors: tetrahydrobiopterin (BH_4) and molecular oxygen. The catalytic activity consists in an electron flow between NOS cofactors where the substrate: L-arginine is oxidized to L-citrulline with concomitant production of NO. Moreover calmodulin (CaM) and Ca^{2+} are essential for functional enzyme [44]. When eNOS is not able to produce NO due the absence of specific cofactors the reduction of oxygen and concomitant production of NO are uncoupled. As consequence of uncoupled eNOS the superoxide anion is produced instead of NO [45]. The importance of eNOS for endothelium homeostasis is related to NO as well as to its side product O_2^-. In physiological conditions NO function is widely characterized as regulator molecule for vasorelaxation and maintenance of healthy vascular beds. However the level of NO is critical for vascular homeostasis. Low and medium NO levels are involved in cellular signaling, while high NO levels are related to apoptosis and cell damage. NO is a gas that diffuse among tissues. NO is a very reactive molecule that spontaneously reacts with free radicals (i.e. superoxide anions) generating reactive nitrogen species (RNS) among which the most common is peroxynitrate (ONOO-). Peroxynitrate is a potent pro-oxidative radical that cause intracellular damage by nitration and S-nitrosylation of proteins, lipid and DNA [46]. Excessive cellular damage causes severe endothelium dysfunctions as reported in multiple cases of cardiovascular disease as diabetes and hypertensions or inflammation [47,48]. eNOS expression and NO are important players for angiogenesis not only in physiological conditions, but also in tumor conditions. NO contributes to angiogenesis by activating intracellular molecular pathway such as the mitogen activated kinases (MAPK), cyclic GMP (cGMP), and by regulating expression of fibroblast growth factor (FGF-2) and controlling the balance between metalloprotease (MMP) and their inhibitors in surrounding tissues. In tumor conditions it has been reported that tumor cells can upregulate NO levels by induction of specific intracellular NOS isoforms (iNOS and nNOS) in order to activate NO-dependent angiogenic signaling. Also eNOS is normally expressed by endothelial tumor cells and is sensitive to multiple factors present in the tumor microenvironment. Pro-angiogenic factors such as vascular endothelial growth factor (VEGF), sex hormones or angiopoietins activate eNOS and positively regulate eNOS in endothelial cells through specific molecular pathways such as 1) Akt-phosphoinositide3 (PI3K) pathway, 2) phospholipase Cγ (PLCγ)-diacylglicerol (DAG)/Ca^{2+} 3) adenilate cyclase (AC) -protein kinase A (PKA). Upregulation of eNOS triggers NO-specific intracellular signaling not only through cGMP, but also leading to post-translational modifications of proteins to form S-nitrosothiol and, thus, generating a specific oxidative signaling mediated by S-nitrosylation. An example of such mechanism is the nitrosylation of caspase 3 that inhibits apoptosis or the nitrosylation of p21Ras that enforces cGMP signaling by increasing endothelial cell proliferation [49,50]. Supporting evidences for NO involvement in tumor progression come from in vivo studies

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with NOS inhibitors that have demonstrated a peculiar role for NO in sustaining tumor growth. Anti-metastatic effects have been reported in several kinds of tumors under treatment with NOS inhibitors N^G-methyl-L-arginine (NMMA) and N^G – Nitro-L-arginine methyl ester (L-NAME) [51,52].

Together with these enzymes the mitochondrial electron transport chain (ETC) has been recognized as responsible for pro-oxidative species production. The mitochondrial respiratory chain is one of the first sources of pro-oxidative species to have been characterized in cells. Mechanism through witch oxidative species are produced in mitochondria are widely described as side products of ETC [53,54]. As it has been described ETC consists in an electron flow among different protein complexes in the inner mitochondria membranes. Electrons from NADPH are transferred NADPH-ubiquinone oxidoreductase complex I which consequently transfer electrons downstream to complex II. Then, electrons according to electrochemical gradients flow to complexes III and IV. The final step of the chain is the reduction of oxygen to water, however it has been quantified that about 1-4% of oxygen fails to be properly reduced and superoxide is produced as consequence. Dysfunctional ETC leads to high levels of ROS in mitochondria that are reported as cytotoxic, however this condition has been also associated with induction of pro-angiogenic signaling [55]. In vitro and in vivo treatments with inhibitors of ETC (i.e. rotenone) inhibits VEGF -induced signaling and vascular walls remodeling [56] suggesting that ETC may play a role in redox signaling in normal and pathological angiogenesis.

4. Cellular systems for counterbalance oxidative species in angiogenesis: Natural antioxidants and scavenging systems

4.1. Antioxidant enzymes

In order to limit oxidative stress levels cells are armed with a series of enzymes and molecules. Important enzymes for degradation of hydrogen peroxide and superoxide are family of superoxide dismutase (SOD), catalase (CAT), peroxiredoxins (PRX), thioredoxin (TRX) and gluthatione peroxidase (GPx). All these enzymes play a critical role in modulation redox signaling.

Superoxide dismutase (SOD) is the most important cellular mechanism of protection against superoxide anion (O_2^-). SOD catalyzes the dismutation of O_2^- into hydrogen peroxide (H_2O_2). The catalytic reaction of SOD involves metal cations (i.e. Cu, Zn, Mn) as cofactors that continuously shift between reduced and oxidized forms in the active site of the enzymes. In humans there are three isoforms of superoxide dismutase enzymes that are distinguished for their cellular localization: SOD1 (CuZn-SOD) which is localized essentially in the cytosol, SOD2 (Mn-SOD) which is localized in the mitochondria and SOD3 (CuZn-SOD, also known as ec-SOD) which is localized in the extracellular matrix. All three isoforms catalyze the same reaction, which is important, not only to scavenge the cytotoxic effects of superoxide anion accumulation (i.e. oxidation and inactivation of proteins), but also to prevent the reaction of

O_2^- with nitric oxide (NO) to generate peroxynitrate. In this way these enzymes guarantee the metabolism of H_2O_2, important for redox signaling [57]. In the vasculature the signaling of H_2O_2 produced by SODs activates multiple pathways important for angiogenesis. The H_2O_2 generated by SOD3 in the extracellular space favorites VEGFR2 signaling and consequently modulates angiogenesis. H_2O_2 produced by SOD3 under conditions of ischemic injury, protects tissues and promotes neovascularization by enhancing Ras-ERK, PI3kinase-Akt pathways and VEGF expression [58].

The H_2O_2 generated by SOD1 is actively produced in endosomes under inflammation signals and activates NF-kB. Moreover such H_2O_2 generated by SOD1 is particularly important in endothelial cells where acts as endothelium–derived hyperpolarization factor (EDHF). It has been demonstrated that in the tumorigenic context, SOD1 overexpression promotes angiogenesis and tumor growth. Also the H_2O_2 generated by SOD2 is important for endothelium. It has been demonstrated that SOD2 overexpression favorites Akt pathway activation and enhances vessels formations *in vivo* by favoring endothelial cells sprouting. On the contrary, SOD2 deficiency causes increased mitochondrial O_2^- that results in mitochondria damage (i.e. mtDNA and mitochondrial proteins oxidation) and endothelial dysfunctions [59,60]. Additionally, all SODs enzymes modulate vessels homeostasis by influencing EPCs. $SOD3^{-/-}$ mice show EPCs failure in physiological processes of migration and differentiation. A recent report indicate that SOD1-deficient EPCs show shortages in migration and ability to generate small vessels networks [61]. Thus, SODs enzymes play their role in redox signaling by regulating angiogenesis through H_2O_2 and protecting EPCs from excess of O_2^-.

Catalase (CAT) catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen by helping the antioxidant machinery in cells. The active site of the enzyme is made by four porphyry heme groups, that are essential to catalyze the flow of electrons between atoms. The mechanism of reaction is not jet fully characterized however it is supposed to occur into two steps: in the first step the iron is reduced with concomitant production of water, in the second step another molecule of hydrogen peroxide enters into the active site and allows the re-oxidation of iron with contemporary production of water and oxygen [62]. The balance of the reaction consists of two molecules of H_2O_2 that are decomposed into two molecules of water and one molecule of oxygen. Catalase is intracellular localized, mainly in peroxisomes of animal and vegetal cells. In addition, there are also data supporting its localization in the mitochondria and cytosol. Catalase is an enzyme shared between all organisms. All cells contain catalase, however knock-out animal models do not display severe phenotypes. Also human patients showing reduced levels of catalase enzymes do not display severe health disorder [63]. It is supposed that the lack of catalytic activity of catalase may be replaced by multiple alternative antioxidant systems. The catalase plays an important role in the redox signaling since it regulates H_2O_2 levels, which is important for homeostasis of vascular beds. Catalase of endothelial cells protects smooth muscle cells from oxidative damage of luminal peroxide [64] and is involved in mechanisms of vessels relaxation [65]. Moreover, it has been also reported that catalase in combination with SOD play a synergistic role in the regulation of endothelium permeability. Overexpression of catalase was also applied to breast cancer cells

in order to down-regulate intracellular ROS levels and make tumor cells more sensitive to therapy (paclitaxel, etoposide and arsenic triosside) [66].

Peroxiredoxins (PRX) are a family of ubiquitous antioxidant enzymes constituted by several isoforms. PRX catalytic activity consists in the reduction of cellular hydrogen peroxide and for some aspects it overlaps with enzyme activity of other antioxidant enzymes (i.e. catalase and GPx) [67]. However, PRXs differentiate from other antioxidant enzymes for their mechanism of activity. Their enzymatic active site is constituted by cysteine amminoacids that metabolize H_2O_2 by cycling between oxidation and reduction reactions. When a molecule of hydrogen peroxide enters, the active Cys-SH oxides into Cys-SOH. This intermediate form can be further oxidized to Cys-SO_2H. The recycling of cysteine is mediated by glutathione, ascorbic acid or sulfiredoxins. According with the setting of the active site PRX are divided into three groups. The first two groups are Typical 2-Cys-PRX and Atypical 2-Cys-PRX, according with folding structure, and both contains two residues of Cys in their active site are. The third class, 1-Cys-PRX contains only one Cys in the active site. Besides the difference in the number of active cysteine, all PRXs act as intracellular H_2O_2 scavengers. PRXs are localized primarily in the cytosol, but they are localized also in intracellular organelles (peroxisomes, mitochondria) where they take part to regulation of H_2O_2 levels and redox signaling [68,69]. In vivo knockout mice for PRX-VI are more sensitive to oxidative stress under hyperoxia exposure, while knockout mice for PRX-I and PRX-II develop severe blood cells disease (hemolytic anemia and hematopoietic cancer) [70,71]. The role of PRXs in redox signaling in cardiovascular system is still not clear.

Thioredoxins (TRX) are a small class of antioxidant enzymes composed of two isoforms: TRX1, which is primarily localized in the cytosol and nucleus, and TRX, which is found in mitochondria. All TRX enzymes are ubiquitously expressed and are characterized by a dithiol-disulfide site. The active site of TRX contains a specific and highly conserved motif with two residues of Cysteine that are essentials to reduce oxidized proteins and buffer ROS. TRX can be continuously reconverted from oxidized form into reduced form thanks to thioredoxin reductase enzymes activity. The TRX system is modulated by an endogenous inhibitor protein, called TXNIP (TRX-interacting protein), that prevents TRX to form disulfides [72]. The TRX system has been shown to be essential for life since the knockout mice of either isoform is lethal for embryo development [73,74]. Moreover endothelium specific *Trx2* transgenic mice as well as mice overexpressing *Trx1* demonstrate a crucial role of this class of enzymes in buffering oxidative stress in endothelial cells. TRX1 can modulate different cellular processes involved with endothelial cell homeostasis and angiogenesis. In endothelial cells TRX1 prevents degradation of HIF1α and consequently modulate VEGF expression facilitating pro-angiogenic processes. Moreover, TRX1 can regulate proliferation and migration of endothelial cells by modulation of NF-kB activity and upregulation of matrix metalloproteases (MMPs). TRX2 have been demonstrated to play also a specific role in endothelial cells by inducing angiogenesis and arteriogenesis in pathological conditions (i.e. murine model of ischemia) [75]. The importance of this antioxidant system for promoting angiogenesis has been considered for development of anticancer drugs. In vitro studies performed with TRX inhibitors (i.e. PMX464, AJM290) confirm the pivotal role of this class of enzymes in preventing endothelial cell proliferation and differentiation [76].

Glutathione peroxidases (GPx) are a family of enzymes that catalyze the reduction of hydrogen peroxide and organic hydroperoxides to water. The reaction consists in the oxidation of monomeric glutathione to glutathione disulfide with the involvement of a selenic acid group. Oxidized glutathione molecules are then reduced by a specific glutathione reductase [77]. In humans there are eight isoforms of glutathione peroxidases with different intracellular localizations and different relative abundance in tissues. Human GPx1, GPx2, GPx3, GPx4 and Gpx6 are different from other isoforms for containing seleno-cysteines in their catalytic sites, which identifies them as seleno-proteins. All GPxs play a fundamental role in the antioxidant molecular network as peroxide scavenging enzymes, however specific notes are reported for different isoforms. GPx4 has been identified mainly as phospholipid hydroperoxidase since it not only reduces peroxides but it is also efficient in reducing phospholipids, cholesterol and lipoproteins hydroperoxide [78]. In pig livers GPx4 activity was reported for inhibition of lipid peroxidation [79] and curiously crucial for sperm maturation [80]. GPx3 is produced in the tubules of kidney and secreted in extracellular fluids as well as in the plasma, but its antioxidant activity does not seem to be essential since $GPx3^{-/-}$ mice do not show abnormal phenotype [81]. Recent studies on GPx3 promoter regulation suggest that its expressivity is implicated in epithelial tumor development but a specific role needs to be addressed [82]. GPx2 is expressed in the gastrointestinal system and is supposed to play a key role as antioxidant enzyme in the gut. Also, $GPx2^{-/-}$ mice do not show abnormal phenotype [83] but in vitro and in vivo data regarding loss of GPx2 expression report a role for GPx2 in regulation of inflammation-mediated carcinogenesis and for supporting growth of established tumors. Among the GPxs isoforms the most studied and characterized is GPx1, which is also the most abundant one. It is ubiquitously expressed and it is localized mainly in the cytosol and in mitochondria. GPx1 is believed to be the most important peroxide scavenger in the family [84], even tough also $GPx1^{-/-}$ mice are not lethal and develop normally [85]. In vivo data indicate that the loss of this enzyme is correlated with high oxidative damage condition. Loss of GPx1 in condition of cerebral inflammation increases pro-oxidative species level and favors interactions between leukocytes and endothelial cells of cerebral microvasculature [86]. Loss of GPx1 in human microvascular cells as well as in $GPx1^{-/-}$ mice favors endothelium response to lipo-polisaccaride pro-oxidant stimuli favoring intracellular reactive oxygen species accumulation and altering expression of adhesion molecules. Levels of GPx1 are also reported to modulate angiogenic endothelial progenitor cells (EPCs) in correlation with aging. EPCs of old subjects, that have impaired GPx1 levels, are more sensitive to oxidative damage [87].

4.2. Antioxidant molecules

Recent evidence suggests that many natural **antioxidant molecules** contained in foods or plants have beneficial effects against tumor progression. Polyphenols as well as terpenoids act on overall oxidative stress levels. By modulation of cytokines, metabolizing enzymes, growth factors and various molecules in redox signaling, antioxidants regulate pathways for tumor angiogenesis. Natural **polyphenols** are a class of compounds constituted by molecules containing repetitive units of phenols that characterize them with antioxidant properties. Polyphenols are naturally present in vegetal derived foods (i.e. fruits, tea, red wine, honey, olive oil) and can be assumed directly with the diet [88]. Data regarding alimentary habits

correlate black tea assumption with beneficial effects on endothelium dysfunctions in individuals with chronic heart disease and hypercholesterolemia [89]. In vitro studies on endothelial cells demonstrate polyphenols modulate redox signaling by regulation of arachidonic acid cascade. In particular, it has been reported that polyphenols from virgin olive oil and red wine reduce significantly angiogenesis by inhibition of cyclooxigenase2 (COX2) and activation of redox sensitive NF-kB pathway [90]. Microarray data and RT-PCR analyses show that treatment of endothelial cells (HUVEC) with resveratrol (contained in red wine) can upregulate eNOS and decrease the levels of endothelin-1, suggesting a protective role against endothelium contractions. Moreover, resveratrol exerts a protective effects on endothelium as assed also under pro-oxidative state (in presence of H_2O_2). Together with anthocyanin, also polyphenols (contained in berries that have red pigments) are reported to have an antioxidant positive effect for cardiovascular system. Recently, anthocyanins from six berries extracts have been mixed in a formula (OptiBerry), which in vitro exhibits anti-angiogenic properties on human microvascular endothelial cells and also in vivo impairs endothelioma cells for tumor growth [91].

Among **lipids** there are also very important antioxidant molecules. They are naturally present in plants like carotenes (retinol and b-carotene), alpha-tocopherol (also known as Vitamin E) or synthetized by animal cells, like CoenzymeQ10. The characteristic lipid character of these molecules allows them to localize in cell membranes (intracellular organelles and plasma membrane) where they can buffer lipid radicals and prevent reactions of peroxidation. Antioxidant Vitamin E properties for lipid peroxidation were efficiently assayed in $GPx4^{-/-}$ mice [92]. Carotenoids are tetraterpenoid pigments contained exclusively in plant cells that can be assumed with diet and act as terminal antioxidant molecules, once oxidized they can not be "re-used" from cells [93]. CoenzymeQ10 (CoQ10) is a terpenoid molecule whose antioxidant activity has been reported for maintenance of healthy cardiovascular system. Recent clinical trials have also show the use of CoQ10 for lowering blood pressure. At the present there are not evidences regarding the involvement of lipid antioxidant molecules in conditions of tumor angiogenesis [94].

Several other **antioxidant genes** are normally induced in cells to shield against dangerous deregulation of redox balance. Among those which play a key role in angiogenesis we can find heme-oxygenase-1 (HMOX-1) and nuclear factor erythroid 2 (NRF2) [95]. Upregulation of these genes are correlates to tumor metastasis and progression suggesting how oxidative stress is a condition implicated in tumor angiogenesis [96].

5. Angiogenic molecules regulated by redox signaling

Vascular Endothelial Growth Factor (VEGF) family encloses six glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D AND VEGF-E, all of them belong to a superfamily of growth factors. Endothelial cells have three types of specific VEGF receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1), VEGFR-3 (Flt-4). Signaling mediated by VEGF and respective receptors has been characterized as one of the most powerful factors for induction and maintenance of

angiogenesis in a series of physiological as well as pathological (i.e. tumor) angiogenesis [97,98]. Among the multiple mechanisms of VEGF signaling regulation we can also found its redox state. Modulators of redox state as the concentration of oxygen directly regulates VEGF levels. VEGF promoter contains a hypoxia responsive element that can be bound by transcription factors HIF-1α- HIF1β under conditions of low oxygen concentration in vascular vessels (hypoxia). In vitro studies by using human genome array on pulmonary artery endothelial cells maintained in hypoxia for 24hours report up-regulation of expression of VEGF genes. Accordingly, in vivo hypoxia conditions induce VEGF and VEGFR-1/2 expression [99,100]. It has been also reported that modulation of redox state by NO levels regulates angiogenesis and tumor progression through modulation of VEGF–VEGFR signaling. In vitro treatments of human tumor cells with NO-donor (i.e. SNAP) or NO-generating compounds upregulate VEGF expression and stimulate angiogenesis [101]. Further specifications about VEGF modulation by NO are related to endothelial NOS activity.

Angiopoietins (Ang) are a group of four growth factors (Ang-1, Ang-2, Ang-3, Ang-4) involved in blood vessels formation. Signaling mediated by angiopoietins and their specific receptors (TIE, tyrosine kinases receptor) has been characterized as key factors in angiogenesis [102]. They are particularly sensitive to endothelium environment since angiopoietins are modulated by pro-oxidative species. In vitro and in vivo studies in endothelial cells report Ang1 is induced by hydrogen peroxide and abrogation of catalase activity relates to low ability in cell migration and vessels formation [103]. Also, angiopoietin-like proteins (ANGPTL) are involved in redox signaling in tumor conditions. Up-regulation of expression of ANGPL4 promotes NADPH oxidases activity causing an alteration in relative abundance of superoxide anion over hydrogen peroxide. Finally, such redox alteration induces tumor cells escape from anoikis and promotes survival via specific activation of PI3K/PKBα/ERK pathway [104].

Vascular Endothelial (VE)-Cadherin is an adhesion protein in the adherent junction complexes of endothelial cells and has been characterized as the major system for controlling endothelial cells junctions [105]. VE-Cadherin controls vascular permeability and remodeling of blood vessels also under mechanical stimuli (shear forces) [106]. VE-Cadherin regulation is sensitive to pro-angiogenic factors, in particular to VEGF [107]. VE-Cadherin is also directly regulated by redox signaling pathway. In vitro assays showed that resveratrol promotes proliferation and migration of cerebral endothelial cells by modulation of VE-cadherin as result of activation of MAPK/ERK pathway and NO upregulation [108]. It has also been demonstrated that resveratrol control initiation of arteriogenesis by blocking oxidative stress dependent phosphorylation of VE-Cadherin [109]. Interestingly, it is also reported that nitrate concentration contributes to control VE-Cadherin stability in adherent junction of human primary endothelial cells (HUVEC) and prevents blood vessel leakage [110].

Nuclear factor-kB (NF-kB) is a transcriptional factor that promotes tumor growth and invasiveness by activation of angiogenic molecules in endothelial cells [111]. Using a zebrafish animal model it has been shown that in vivo NF-kB inhibition causes loss of vascular integrity and interferes with physiologic vessels morphology [112]. Inhibition or negative modulation

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of NF-kB is considered an alternative approach to block pathological angiogenesis. Among inhibitors of NF-kB it has been reported evidence for antioxidant molecules. In vitro treatments of cells with pro-oxidative species (hydrogen peroxide, LPS, TNF-α) activates NF-kB, while contemporary antioxidants addiction inhibit its response [113,114]. NF-kB activation can also be impaired by N-acetylcysteine (that acts in the NO pathway), terpenoids (vitamin E) and mitochondria-specific antioxidant (rotenone) [115,116]. At the present it is not fully clarified as antioxidants interact with NF-kB and inactivate it. It is supposed they can act indirectly by altering different molecules that interact with NF-kB on redox pathways or directly by inhibition of IKK kinase activity [117].

6. Conclusion: Manipulating redox signaling as anti-tumor angiogenesis therapy

Increased generation of reactive oxygen species (ROS) and an altered redox status have long been observed in cancer cells, and recent studies suggest that this biochemical property of cancer cells can be exploited for therapeutic benefits. Cancer cells in advanced stage tumors frequently exhibit multiple genetic alterations and high oxidative stress, suggesting that it might be possible to preferentially eliminate these cells by pharmacological ROS insults [118].

Reactive oxygen species (ROS) might function as a double-edged sword in endothelial cells. A moderate increase of ROS may promote cell proliferation and survival. However, when the increase of ROS reaches a certain level (the toxic threshold), it may overwhelm the antioxidant capacity of the cell and trigger cell death. Under physiological conditions, normal endothelial cells maintain redox homeostasis with a low level of basal ROS by controlling the balance between ROS generation (pro-oxidants) and elimination (antioxidant capacity). Endothelial cells in normal vessels can tolerate a certain level of exogenous oxidative stress owing to their 'reserve' antioxidant capacity, which can be mobilized to prevent the ROS level from reaching the cell-death threshold. In In endothelial cells of tumor vessels the increase in ROS generation from metabolic abnormalities and oncogenic signaling may trigger a redox adaption response. This response leads to an upregulation of antioxidant capacity and a shift of redox dynamics that maintain the ROS levels below the toxic threshold. As such, tumor angiogenic cells would be more dependent on the antioxidant system and more vulnerable to further oxidative stress induced by exogenous ROS-generating agents or compounds that inhibit the antioxidant system. A further increase of ROS stress in these cancer cells using exogenous ROS-modulating agents is likely to cause elevation of ROS above the threshold level, leading to cell death. This might constitute a biochemical basis to design therapeutic strategies to selectively kill tumor angiogenic cells using ROS-mediated mechanisms [119-121].

The role of redox signaling in tumor angiogenesis is not yet completely characterized. Although converse mechanisms are postulated about how oxidative species recruit new blood vessels for tumor progression, it is well established redox signaling modulates angiogenesis. Analysis and characterization of molecules that sustain redox signaling is a new opportunity for set up innovative strategies of anti-cancer therapy (Figure 1).

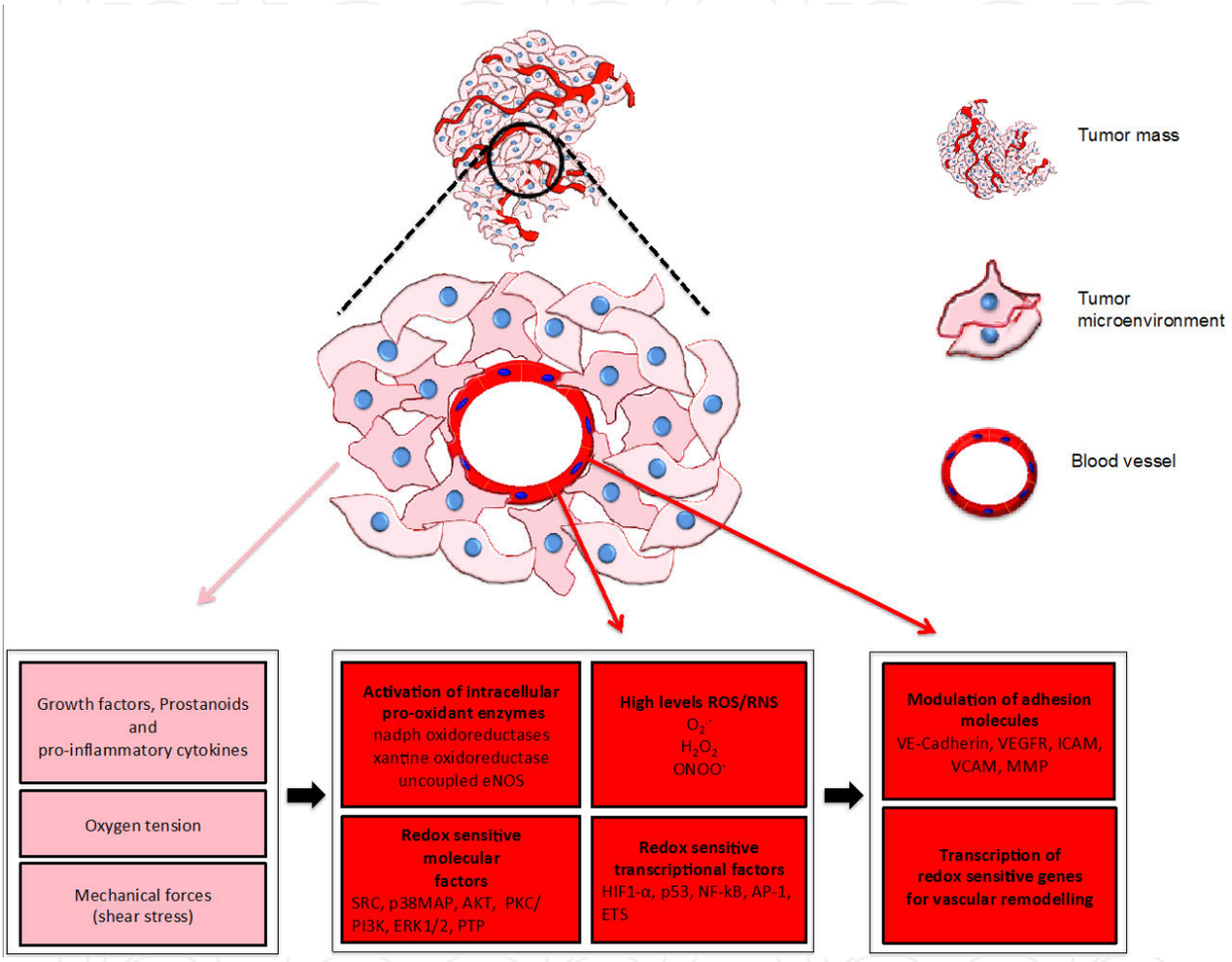

Figure 1. Schematic representation of redox mechanisms in tumor angiogenesis. Multiple stimuli coming from tumor microenvironment (growth factor, prostanoids, oxygen tension, mechanical forces) induce specific activation of intracellular pro-oxidant enzymes (NADPH oxidase, xantine oxidoreductase, uncoupled eNOS). Consequently, raising levels of oxygen and nitrogen pro-oxidant species (ROS/RNS) modulate the activation of multiple cellular pathways by acting on molecular and transcriptional factors. Signaling induced by oxidative species results mainly in endothelial cells motility and proliferation towards vascular remodeling and formation of new blood vessels. HIF-1α hypoxia-inducible transcription factor; ETS E-twenty six family transcription factor; AP-I activator protein 1; p53 tumor suppressor protein; NF-kB nuclear factor – kB. PKC protein kinase C; PI3K phosphatidylinositol3-OH kinase; PTP protein tyrosine phosphatase; SRC tyrosine protein kinase; p38MAPK p38 mitogen-activated protein kinase; Akt serine/threonine-specific protein kinase; ERK1/2 extracellular signal-regulated kinases; MMP matrix metalloproteinase; VE–Cadherin: vascular endothelial-cadherin; VEGFR vascular endothelial growth factor receptor; ICAM intracellular adhesion molecule 1; VACAM vascular cell adhesion molecule 1.

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