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Oxidases and reactive oxygen species during hematopoiesis: a focus on megakaryocytes

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Abstract

Reactive oxygen species (ROS), generated as a result of various reactions, control an array of cellular processes. The role of ROS during megakaryocyte (MK) development has been a subject of interest and research. The bone marrow niche is the major site of MK differentiation and maturation. In this environment, a gradient of oxygen tension, from normoxia to hypoxia results in different levels of ROS, impacting cellular physiology. This article provides an overview of major sources of ROS, their implication in different signaling pathways, and their effect on cellular physiology, with a focus on megakaryopoiesis. The importance of ROS-generating oxidases in MK biology and pathology, including myelofibrosis, is also described.

Keywords

megakaryocytes; oxidases; reactive oxygen species; bone marrow

Introduction

Reactive oxygen species (ROS) may be beneficial for the organism, as in the classic example of ROS produced by nicotinamide adenine dinucleotide phosphate (NADPH) for defense against pathogens. They may also arise as harmful by-products of other oxidative reactions. ROS levels influence a number of basic physiological processes, ranging from cell differentiation and proliferation to cell death. The underlying mechanisms for these functions have been gradually elucidated.

There has been increasing interest in the role of ROS and oxygen stress in the regulation of hematopoiesis. A large body of studies has shown that control of intracellular levels of ROS is essential for maintenance of quiescence and self-renewal potential of hematopoietic stem cells (HSCs). The bone marrow (BM) niche seems to be an essential component of the regulation of ROS in HSCs. Like HSCs, megakaryocytes (MKs) differentiate and mature within the BM niche. These cells undergo a unique cell cycle termed endomitosis, during which the DNA content of the cell replicates without corresponding cell divisions, before

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platelets are released into the circulation. The present article seeks to provide an overview of the recent literature concerning the effects of ROS on different stages of MK development (Figure 1). Although not a focus of this review, important aspects of HSC biology with parallels in the MK lineage will be noted for reference and analysis. More detailed information on the role of ROS in HSCs and other lineages is available elsewhere (Eliasson and Jonsson, 2010; Ghaffari, 2008; Sardina *et al.*, 2011; Suda *et al.*, 2011).

ROS and oxidases

Physiological processes in organisms involve oxidizing/reducing reactions (donation/gaining of an electron). ROS can be generated as a result of those reactions. ROS are free radicals (highly reactive molecules with unpaired electrons in their highest atomic orbital) containing partially reduced forms of molecular oxygen. Examples of ROS include highly reactive oxygen radicals, such as superoxide anions ($O_2{\cdot}^-$), hydroxyl (OH·), peroxyl ($RO_2{\cdot}$) and alkoxyl (RO·) radicals, and non-radicals that are oxidizing agents and/or easily converted into radicals, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Bedard and Krause, 2007). Although H_2O_2 is not a free radical (having no unpaired electrons), it is treated as one because it can easily give rise to reactive hydroxyl radicals (Boonstra and Post, 2004).

Sources of ROS can be either extracellular (pollutants, tobacco smoke, ultraviolet radiation, ionizing radiation, iron salts) or intracellular (mitochondrial respiratory chain, NADPH oxidase complexes, xanthine oxidases (XOs), amine oxidases, nitric oxide synthases (NOSs), myeloperoxidase (MPO), peroxysomes, and metabolism of arachidonic acid by lipoxygenases and cyclooxygenases (Bedard and Krause, 2007; Stocker and Keaney, 2004; Turrens, 2003).

The mitochondrion is the major intracellular source of ROS. Energy production through the mitochondrial respiratory chain is mediated by five enzyme complexes that oversee the reduction of oxygen to water, one electron at a time. Complex IV (cytochrome oxidase) retains all partially reduced intermediates until full reduction is achieved. However, the Q-cytochrome c oxireductase (Complex III), as well as the nicotamide adenine dinucleotide quinone (NADH-Q) reductase (Complex I), are well-documented sources of ROS, as they may leak electrons to oxygen, partially reducing this molecule to superoxide anion ($O_2{\cdot}^-$) (Kowaltowski *et al.*, 2009; Turrens, 2003). Apart from the mitochondria, membrane-bound NADPH-oxidase (NOX) induces production of ROS in phagocytes, which is important for defense against pathogens (Rossi and Zatti, 1964). XOs are key enzymes in purine metabolism catalyzing oxidative reactions to produce uric acid, which is an antioxidant and free-radical scavenger (Stocker and Keaney, 2004). Nitric oxide synthases (NOS) produce the free radical NO from oxidation of L-arginine. Three isoenzymes of NOSs are known: nNOS, expressed in neuronal tissues; eNOS, expressed in endothelial cells; and iNOS, the inducible form expressed in response to cytokines or bacterial products. The inducible form of NO is involved in proinflammatory reactions, functioning as a cytostatic and cytotoxic molecule (Knowles and Moncada, 1994). MPO is produced in myeloid phagocytic cells and its primary function is the destruction of microorganisms. The oxidation of chloride by MPO and H_2O_2 results in HOCl, a highly reactive oxidizing agent (Klebanoff, 2005). Breakdown of fatty acid chains by β-oxidation in peroxisomes is another source of intracellular ROS (Boonstra and Post, 2004). Lipoxygenases catalyze the insertion of molecular oxygen into polyunsaturated fatty acids to give rise to active lipids, including prostaglandins, thromboxanes, and leukotrienes (Kuehl and Egan, 1980).

Antioxidants

Although ROS are important for normal physiology, accumulation of ROS at high levels can be harmful to the organism, causing damage to a variety of biomolecules, such as lipids, DNA, carbohydrates and proteins. Consequently, a number of defense systems have evolved to prevent such damage. However, under conditions of increased accumulation of ROS, these defense mechanisms are not always sufficient to regulate the intracellular ROS balance, leading to oxidative stress with detrimental effects on cellular homeostasis.

The antioxidant systems in mammalians include enzymes, such as superoxide dismutases (SOD), glutathione (GSH), glutathione peroxidase (GPX), glutashione S-transferases (GST), catalase, and peroxiredoxins (Prx). SOD2 has been reported to affect the erythrocyte lineage; no other effects of SODs have been reported in hematopoiesis. GSH is the hallmark redox buffer in living cellular systems. GSH is a tripeptide of glutamic acid, cysteine and glycine and is the predominant non-protein thiol in biological systems. The balance between reduced glutathione (GSH) and oxidized glutathione (GSSG) is critical in maintaining redox homeostasis (Grek *et al.*, 2011). GPX cooperates with catalase and uses GSH for the conversion of H_2O_2 into water and GSSG. GPX requires selenium for catalytic activity. GST is another member of the glutathione-dependent antioxidant defenses, catalyzing the thioester conjugation to glutathione. TLK 199 or Telintra is a peptidometic inhibitor of GST, which recently showed promising results for treatment of myelodysplastic syndrome (MDS) (Grek *et al.*, 2011). Prxs are a family of small antixodant proteins characterized by an amino-terminal catalytic cysteine residue that is converted to sulfenic acid via reaction with H_2O_2. Deficiency of Prx I and II in mice affects erythroid homeostasis; loss of Peroxiredoxin I further increases susceptibility to several malignancies (Ghaffari, 2008). Non-enzymatic molecules, including vitamin E, vitamin C and flavonoids act as antioxidants capable of neutralizing ROS (Halliwell, 1999).

Overview of megakaryopoiesis

MKs are highly specialized blood cells residing primarily in the BM but also in the spleen and lung capillaries (Kaushansky, 2008; Slater *et al.*, 1983). These cells are responsible for the production of platelets, which are renewed on a daily basis (Kaushansky and Drachman, 2002). In adults, MKs derive from BM-residing HSCs. In response to physiological demand, the HSC gives rise to early progenitor cells including the early common myeloid progenitor (CMP); further lineage specification leads to development of the bipotential megakaryocyte/erythrocyte progenitor (MEP) (Akashi *et al.*, 2000). Under certain cytokine stimulations, the bipotential MEP can develop into a highly proliferative early MK-burst-forming unit (MK-BFU) followed by maturation to a colony-forming unit-MK (CFU-MK) (Deutsch and Tomer, 2006). These MK progenitors eventually lose their proliferative ability and commit to an endomitotic cycle. This process leads to mature polyploid MKs that grow several-fold in size. Mature MKs can obtain a DNA content of up to 128N, with the average being 16N (Kaushansky, 1999; Tomer, 2004). During this process, MKs increase the production of proteins necessary for platelet biogenesis and function (Paulus, 1970). Mature MKs form proplatelet extensions that fragment and give rise to platelets (Italiano *et al.*, 2007). Figure 1 illustrates the above described major steps in MK development.

Thrombopoietin (TPO), also known as c-Mpl ligand or MK growth and development factor (MGDF), is the key regulator of megakaryopoiesis. In pathological conditions, such as thrombocytopenia, where there is high demand for platelets, TPO release to the MK microenvironment is increased (Sungaran *et al.*, 2000; Sungaran *et al.*, 1997). Mutations in the regulatory regions of the TPO gene that result in overexpression of TPO, as well as activating mutations of the c-Mpl receptor, are associated with familial essential

thrombocythaemia, characterized by enhanced megakaryocyte progenitor proliferation and platelet production (Deutsch and Tomer, 2006).

TPO is not the sole regulator of MK development. A number of cytokines including platelet-derived growth factor (PDGF) are involved is this process as well. Earlier studies in primary murine and human BM cultures, as well as in human umbilical cord blood $CD34^+$ cells, have shown that PDGF-BB is a positive regulator of megakaryopoiesis (Su *et al.*, 2001; Su *et al.*, 2005; Yang *et al.*, 1995). Interestingly, PDGF-BB upregulates the expression of MK-associated transcription factors, such as c-Fos, GATA-1 and NFE2 in megakaryocytic cell lines (Chui *et al.*, 2003). Targeted deletion of the PDGF-B polypeptide chain is embryonic lethal. Hematological analysis of these embryos reveals erythroblastosis, macrocytic anemia, and thrombocytopenia (Kaminski *et al.*, 2001; Leveen *et al.*, 1994).

MK differentiation: significance of hypoxia in the BM niche

HSCs predominantly remain in a quiescent state in the low-oxygen environment of the osteoblastic niche (Adams and Scadden, 2006; Parmar *et al.*, 2007). In contrast, HSCs and other progenitor cells actively undergo cell division for proliferation and further differentiation in the more oxygenic vascular niche (Kopp *et al.*, 2005; Li and Li, 2006). Thus, an oxygen gradient from below 1% in the most hypoxic environment to 6% in the sinusoidal cavity provides conditions that allow different regulatory processes to take place, including self-renewal and differentiation (Eliasson and Jonsson, 2010). A study by Jang *et al.* indicates that HSCs that produce lower levels of ROS are more primitive, as shown by higher self-renewal capacity. On the other hand, ROS^{high} HSCs exhibit significant exhaustion in serial transplantation assays, and have increased levels of p38 MAPK and mTOR. Importantly, treatment with a p38 inhibitor or rapamycin (mTOR inhibitor) was able to restore HSC function in the ROS^{high} population (Jang and Sharkis, 2007). Pallota et al., (2009) developed an *in vitro* system combining human osteoblasts (hOST) and hypoxia to better model the BM osteoblastic niche. Differentiation of human $CD34^+$ cells to MKs in this system revealed that in hypoxic (5% O_2) but not in normoxic (20% O_2) conditions, there is a progressive increase in type I collagen in the culture, demonstrating the requirement of hematopoietic progenitors for matrix deposition and modulation of the niche environment in hypoxic conditions. Moreover, hOST had an inhibitory effect on MK maturation and proplatelet formation, which was further exacerbated by hypoxia (Pallotta *et al.*, 2009).

MKs grow and mature in proximity to the BM sinusoid space where there is increased oxygen tension (Junt *et al.*, 2007; Li and Li, 2006). There, MKs shed platelets into the sinuses, the microvascular structures within the BM space (Junt *et al.*, 2007). MKs are also present in another compartment of high oxygen concentration, the lung capillaries (Zucker-Franklin and Philipp, 2000). Since MKs mature in sites of high oxygen level relative to the average 5% pO_2 in the BM hematopoietic niche (8% pO_2 in the BM sinusoids and 16% pO_2 in the lung capillaries), it is reasonable to hypothesize that oxygen levels contribute to MK maturation (Kietzmann *et al.*, 1999; Pennathur-Das and Levitt, 1987). One of the earliest studies on oxygen tension during MK maturation showed that $CD34^+$ peripheral blood (PB) cells gave higher numbers of $CD41^+$ MKs under 20% pO_2 than under 5% pO_2 conditions. The TPO-induced increase in MK size was also greater under 20% pO_2 culturing conditions (LaIuppa *et al.*, 1998). MK differentiation under 20% pO_2 also yielded higher ploidy MKs as well as platelet forming MKs. In contrast, 5% pO_2 conditions gave greater numbers of CFU-MK (Mostafa *et al.*, 2000). This increase in MK maturation, ploidy and terminal differentiation under 20% O_2 was also associated with elevated expression of MK maturation-specific transcription factors GATA-1 and NFE2. In addition, the level of E2F-1, a ubiquitously expressed cell cycle transcription factor, was increased by day 6 under 20% pO_2 culturing conditions (Mostafa *et al.*, 2001).

Collectively, these studies indicate that the hypoxic environment in the BM niche is favorable for maintenance of more immature MK progenitors while higher oxygen tension is essential for complete MK maturation and platelet production (Figure 2).

TPO and PDGF signaling and MK expansion: the role of ROS as signaling moieties

Fluctuating low levels of ROS are shown to be important in a number of regulatory processes. More specifically, H_2O_2 can serve as a second messenger promoting cell proliferation (Martindale and Holbrook, 2002). Although controversial, the notion of ROS as activators of signaling processes is slowly gaining ground (D'Autreaux and Toledano, 2007).

With respect to the mechanisms involved in the effect of ROS on proliferation, one study points to the possibility of H_2O_2 and $O_2\cdot^-$ being generated at sub-micromolar levels by cells in response to cytokine stimuli. These may act as signaling mediators, thus promoting growth responses. Indeed, hematopoietic growth factors that stimulate proliferation and differentiation of HSCs and progenitor cells signal through ROS. Studies in MO7e human megakaryoblastic leukemia cells showed that growth factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3(IL-3), stem cell factor (SCF) and TPO induce a rapid increase in ROS levels (Prata *et al.*, 2004). This peaking of ROS, in turn, may be essential for the activation of cytokine receptors.

The majority of signaling responses occur through the phosphorylation of tyrosine residues. Seminal works demonstrated that treatment of cells with H_2O_2 induces a global increase in proteins with phosphorylated tyrosine residues (pTyr), thus establishing a correlation between ROS and signaling activation (Heffetz *et al.*, 1990). In vascular smooth muscle cells (VSMC) treated with PDGF, the time-course of H_2O_2 production was similar to that of PDGF-induced tyrosine phosphorylation.

Further studies aimed at discovering the proteins phosphorylated in response to ROS. In MO7e cells, the β common chain receptor for both GM-CSF and IL-3 is phosphorylated upon H_2O_2 stimulation, as upon stimulation with GM-CSF. H_2O_2 stimulation also induced tyrosine phosphorylation of PDGF α- and β-receptors (Gonzalez-Rubio *et al.*, 1996). Addition of the antioxidant pyrrolidine dithiocarbamate (PDTC) to the cultures decreases the ROS levels and inhibits tyrosine phosphorylation induced by GM-SCF (Sattler *et al.*, 1999). Mediators of signaling responses are also phosphorylated in response to ROS production. STAT5, which is a mediator of TPO responses, is phosphorylated in response to H_2O_2 stimulation (Sattler *et al.*, 1999). Inhibition of ROS production hampered the activation of serine/threonine protein kinase (AKT), signal-transducer and activator of transcription 3 (STAT3) and STAT5 in an HEL cell line stimulated with TPO (Sardina *et al.*, 2010). Other studies have focused on the activation of mitogen-activated protein kinases (MAPKs). This can be achieved by direct extracellular-regulated MAP kinase (ERK) phosphorylation and activation, where cells are exposed to H_2O_2, or indirectly by activating upstream effectors such as MAP kinase/ERK kinase (MEK) (stimulated by $ONOO^-$ or H_2O_2), Raf-1, or protein kinase C (PKC) (stimulated by H_2O_2) (Abe *et al.*, 1998; Zhang *et al.*, 2000). As a result, ROS-mediated MAPK activation can induce upregulation of transcription factors, such as c-fos and c-jun (Buscher *et al.*, 1988; Rao, 1997). ERK activation in K562 and HEL cells occurs during phorbol myristate acetate (PMA)-induced MK differentiation, and this activation is hampered by inhibitors of ROS production (Sardina *et al.*, 2010).

Signaling initiated by protein phosphorylation induced by tyrosine kinases is followed by de-phosphorylation by protein tyrosine phosphatases (PTPs) for adequate termination of the

signaling. Recent studies indicate that perhaps the major role of ROS in cell signaling is the modulation of PTP function. The signature motif of the PTP superfamily contains an invariant Cys residue, which is important to catalytic activity and is particularly susceptible to oxidation (Tonks, 2005). The classical example is the MAP kinase phosphatase-3 (MKP-3), a specific regulator of Erk, oxidized in vitro by H_2O_2 (Kamata *et al.*, 2005). The low molecular weight protein tyrosine phosphatase (LMW-PTP) negatively regulates PDGF signaling through binding and dephosphorylation of the receptor. LMW-PTP is oxidized and inactivated by exposure to H_2O_2 or to NO; this oxidization can also be effected by PDGF signaling, likely through H_2O_2 production. Moreover, glutathione, an antioxidant, most likely regulates the reversibility of LMW-PTP inactivation (Chiarugi, 2001). Exposure of cells to H_2O_2 or PDGF induces Akt phosphorylation and oxidation of phosphatase and tensin homolog (PTEN) (Kim *et al.*, 2011). The antioxidant PrxII, has been demonstrated to locally regulate PDGF signaling in VSMC by decreasing the oxidation of PTPs, thereby functioning as a negative regulator of PDGF signaling (Choi *et al.*, 2005).

IL-3 and erythropoietin (Epo) induce a transient increase in ROS levels when added to cultures of the hematopoietic progenitor cell line 32Dcl3. Furthermore, treatment of these cells with the antioxidant N-acetyl-L-cysteine (NAC) inhibits the IL-3-induced phosphorylation of JAK2, AKT and ERK. Moreover, upon treatment with the antioxidant, there is a downregulation of cyclin D2 and cyclin E concomitantly with an increase in expression of the cell cycle inhibitor p27, thus inhibiting G1 to S phase progression (Iiyama *et al.*, 2006).

Studies in HeLa cells and primary fibroblasts demonstrate that exposure of cells to H_2O_2 or peroxynitrite induces Akt activation respectively mediated by epidermal growth factor (EGF) and PDGF receptors (Klotz *et al.*, 2000; Wang *et al.*, 2000). Other pathways activated by ROS are the p38 and c-Jun N-terminal kinases (JNK). These pathways are responsive to oxidative stress and are associated with apoptosis and mitotic arrest (Martindale and Holbrook, 2002).

The above ROS-affected signaling processes are clearly involved in expansion of primary BM MKs, as STAT and MAPK activation are important for MK proliferation (Miyakawa *et al.*, 1996; Severin *et al.*, 2010), and so are G1 phase cyclins (Eliades *et al.*, 2010; Zimmet *et al.*, 1997). Although the accumulated evidence is not sufficient to establish a definitive role of ROS in signaling affecting MK biology, the data indicates a strong effect of ROS on the activation of tyrosine kinase signaling and MK expansion. For PDGF signaling, there is a need to validate current findings in the megakaryocyte lineage. Further studies elucidating the precise mechanisms of this effect are awaited.

MK endomitosis and polyploidization: role of ROS and NAPDH oxidases

As described above, a number of studies have investigated the role of ROS in MK maturation. Therefore, it is of interest to determine the source of ROS in this lineage. An important insight came from the observation that diphenylene iodonium chloride (DPI), an inhibitor of flavoprotein-dependent enzymes, inhibited platelet aggregation (Salvemini *et al.*, 1991). NADPH-oxidase is such an enzyme, and also a known enzymatic source of ROS (Rossi and Zatti, 1964). The family of NADPH-oxidase or NOX proteins consists of oxidases responsible for the transfer of electrons across biological membranes (Bedard and Krause, 2007). Members of the family include the phagocyte NOX2 ($gp91^{phox}$), NOX1, NOX3, NOX4, NOX5 and the dual oxidases DUOX1 and DUOX2 (Bedard and Krause, 2007). Organizer subunits $p47^{phox}$ and NOXO1 as well as activator subunits $p67^{phox}$ and NOXA1 and modulator subunit p40 associate with NOXs (Bedard and Krause, 2007; Geiszt,

2006). All members of the NOX family contain six transmembrane domains, two binding sites for heme and conserved binding sites for NADPH and flavin (Geiszt, 2006).

Recent studies have focused on the source of ROS in HSCs. In PB-derived $CD34^+$ cells, a low mitochondrial oxygen consumption rate was detected, thus qualifying HSCs as a poor oxidative phosphorylating cell type. In addition to low mitochondrial oxygen consumption, the authors depicted the contribution of NOX2 and NOX4 in extra-mitochondrial oxygen consumption (Piccoli *et al.*, 2005). Moreover, both catalase and the NOX pharmacological inhibitors, Apocynin and DPI, inhibited ROS production by human BM-derived HSCs. These cells were also shown to express at least three different NOX isoforms - NOX1, NOX2 and NOX4 - at both the mRNA and protein level along with a set of their regulatory subunits (Piccoli *et al.*, 2007b). Interestingly, $CD34^+$ and $CD133^+$ HSCs express hypoxia-inducible factor 1α (HIF-1α) under normoxic conditions and its levels are positively regulated by NOX-dependent production of ROS (Piccoli *et al.*, 2007a). Seno *et al.* (2001), using a pharmacological approach to target NOXs, –demonstrated that they are possible sources of ROS in human platelets and in the megakaryocytic cell line MEG01. The authors also verified the expression of the NOX regulatory components $p22^{phox}$ and $p67^{phox}$ in both platelets and MEG01cells (Seno *et al.*, 2001).

More recently, it was reported that Nox1 is the major Nox expressed in primary mouse megakaryocytes and contributes to the production of ROS in $CD41^+$ MKs. Inhibition of Nox1 by Apocynin or DPI reduced polyploidization in wild-type MKs. This defect was due to reduced levels of G1 cyclins D3 and E, which have been shown to be important for MK polyploidy (Eliades *et al.*, 2010; Wang *et al.*, 1995). Thus, the effect of ROS on expression of G1 phase cyclins and G1-S cycle progression, along with the reported effect of NOX4 in VSMC polyploidy (McCrann *et al.*, 2009b), point to NOX as an important player in polyploidization. The influence of ROS on G1 phase cyclins has been validated not only in MKs. NOX1-mediated increase in ROS in mouse lung epithelial cells promotes phosphorylation of Erk1/2 and expression of cyclin D1. Antioxidant treatment with catalase or diphylene iodonium, a potent NOX inhibitor, downregulates the levels of cyclin D1 (Ranjan *et al.*, 2006). ROS level is known to increase as cells progress from G1 to S phase and this accumulation is required for entry into S phase (Havens *et al.*, 2006).

MK maturation and platelet formation: the role of ROS

Endogenous ROS are detected in MKs from mouse BM (McCrann *et al.*, 2009a). TPO induces a rapid increase in ROS that is necessary for the megakaryocytic differentiation of human HSCs (Sardina *et al.*, 2010). ROS can control gene expression through the activation of redox-sensitive transcription factors, which in turn coordinate the expression of a number of downstream target genes with antioxidant roles. The classical example is the NF-κB transcription factor, activated by H_2O_2 and intermediates of oxygen radicals (Schmidt *et al.*, 1996; Schreck *et al.*, 1991). p45NF-E2 is a member of the cap ‘n’ collar-basic leucine zipper family of transcriptional activators, with a restricted expression in hematopoietic cells. This family is known to bind antioxidant response elements (ARE) in DNA, which are responsible for the expression of genes related to the defense against oxidative stress. Mice deficient for p45NF-E2 died in neonatal period due to complications of hemorrhage. Absolute thrombocytopenia was detected in those animals. MK ploidization was not affected, indicating that the lack of platelets was due not to differentiation defects in MKs, but rather to a defect in formation of platelet territories in the cytoplasm of MKs (Shivdasani *et al.*, 1995). Recently, work by Motohashi *et al.*, (2010) revealed that NF-E2 p45–promotes ROS accumulation that results in enhanced MK maturation. This is achieved by competition with Nrf2, a key activator of stress-responsive genes (Motohashi *et al.*, 2010).

MK senescence and autophagy: the role of ROS

In conditions of hyperoxia and elevated ROS, cells resort to a decrease in proliferation, cell cycle arrest and senescence (Boonstra and Post, 2004; Shao *et al.*, 2011). Hyperoxia increases the levels of p21, a cell cycle inhibitor that belongs to the family of Cip/Kip proteins (Cazzalini *et al.*, 2010). This increase is mediated by p53 (Helt *et al.*, 2001). In HSCs, senescence can be induced by activation of p38 MAPK (Ito *et al.*, 2006). Recently, studies on UT-7/TPO cells treated with TPO have suggested that cell cycle arrest and senescence participate in the process of MK maturation. This process was accompanied by up-regulation of the senescence marker p21, and mediated by phosphorylation of ERK (Besancenot *et al.*, 2010).

Autophagy is a catabolic pathway in which cells sequester organelles such as mitochondria into lysosomes for degradation. The process of autophagy is an important cellular survival mechanism, which can be activated in response to multiple physiological conditions, including starvation, hormonal imbalance and oxidative stress (Watson *et al.*, 2011). The mammalian target of rapamycin (mTOR) is the central molecular component of autophagy. The Atg1-13 protein complex initiates autophagy, activated by the absence of signaling from mTOR. Mice deficient for Atg7 in the hematopoietic system develop myeloproliferation/MDS and exhibit high mitochondrial content and ROS in HSCs (Mortensen *et al.*, 2011). Interestingly, autophagy has been observed in the megakaryocytic differentiation of the K562 cell line (Colosetti *et al.*, 2009), and mTOR is a reported as a regulator of MK differentiation (Drayer *et al.*, 2006; Raslova *et al.*, 2006); these findings collectively suggest the involvement of ROS-dependent autophagic processes in MK.

Other effects of ROS in MKs

15d-PGJ$_2$

One of the functions of 15d-PGJ$_2$, a J-type prostaglandin, is generation of ROS. 15d-PGJ$_2$ treatment of primary human MKs induced formation of ROS and increased platelet production. Treatment with antioxidants attenuated the platelet-producing effect of 15d-PGJ$_2$ (O'Brien *et al.*, 2008).

TLR2

Stimulation of the Toll-like receptor 2 (TLR2) using the agonist Pam3CSK4 increased production of ROS in the Meg-01 cell line and affected MK-related signaling and gene expression, as well as ploidy of mouse MKs *in vitro* and *in vivo* (Beaulieu *et al.*, 2011).

NO

The addition of NO donors sodium nitroprusside (SNP) or (Z)-1-(2-(aminoethyl)-N-(2-ammonioethyl)amino)diazen-1-ium-1,2-diolate (DETA/NO) to cultures of human CD34$^+$ cells has toxic effects on both total cell number and TPO-induced MK differentiation (as measured by total number of MK and percentage of CD41 expression) (Schattner *et al.*, 2000). The most likely effect of NO on MKs is induction of apoptosis, as demonstrated in detail in megakaryocytic cell lines (Battinelli and Loscalzo, 2000) and MKs derived from human CD34$^+$ cells (Schattner *et al.*, 2000). Stimulation of CD34$^+$ cells with tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) increased endogenous NO levels and suppressed MK growth (Schattner *et al.*, 2000), whereas treatment with TPO suppressed the induction of apoptosis by NO (Battinelli and Loscalzo, 2000). These findings indicate that NO is a mediator of apoptosis in the MK lineage.

ROS in MK pathology

Although there are no reports associating ROS with specific megakaryocytic disorders, oxidative stress has been implicated in a variety of BM failure conditions, such as MDS and myelofibrosis, in which platelet deficiency requires therapeutic intervention.

ROS in MDS

MDS is a clonal stem cell disorder characterized by ineffective maturation of the erythroid, granulocytic, or megakaryocytic lineage. The natural history of the disease is progression from cytopenia to myeloid leukemia. MDS is a disease of the elderly, with a mean age of 70 years at diagnosis (Corey *et al.*, 2007). Age itself is an established critical factor for accumulation of oxidative damage in HSCs (Finkel and Holbrook, 2000). Moreover, accumulating evidence has suggested a major causative role for ROS in the pathogenesis of MDS (Farquhar and Bowen, 2003), beginning with a seminal study that demonstrated the presence of oxidative DNA damage in $CD34^+$ cells from MDS patients (Tauro *et al.*, 2001). A high superoxide concentration has also been detected in supernatant from MDS stroma compared to normal stroma (Tauro *et al.*, 2001). Exposure to cigarette smoke and benzene are reported to be associated with risk of MDS. The progression to disease is also likely to be associated with a deficiency in the detoxification system, such as the NAD(P)H:quinine oxidoreductase (NQO1) deficiency in the hematopoietic stem cells (Rothman *et al.*, 1997). An experimental model of cigarette smoke exposure in guinea pigs demonstrated the correlation between NQO1 deficiency, cigarette smoke exposure, and progression to MDS (Das *et al.*, 2011). Moreover, as noted in our discussion of senescence and autophagy, mice deficient for Atg7 progress to a myeloproliferative disorder/MDS (Mortensen *et al.*, 2011). Mitochondria are the major source of ROS within the cell. Signs of defective mitochondrial autophagy have been observed in erythroid precursors of MDS patients (Houwerzijl *et al.*, 2009), and mutations of mitochondrial DNA are commonly detected in MDS patients (Gattermann, 2004). However, the precise role of autophagy in MDS is still under discussion (Watson *et al.*, 2011).

Lysyl oxidase (LOX) in myelofibrosis

LOX is a copper-dependent amine oxidase that catalyzes the oxidative deamination of lysine and hydroxylysine residues on collagen and elastin precursors. The resulting semialdehydes form covalent cross-linkages, thus stabilizing the extracellular matrix fiber deposits (Lucero and Kagan, 2006). LOX is synthesized as a 50 kDa glycosylated precursor (pro-LOX) which is then secreted and undergoes proteolytic cleavage by pro collagen C-proteinases, including bone morphogenetic protein 1 (BMP1), to release a catalytically active 30 kDa enzyme (LOX) and an 18 kDa propeptide (LOX-PP) (Kagan and Li, 2003; Trackman *et al.*, 1992). The importance of this oxidase was demonstrated in LOX knockout mice, which die soon after birth due to aortic rupture, incomplete diaphragm development and cardiovascular dysfunction (Maki *et al.*, 2002). Studies from our group and others have shown that LOX mobilizes monocytes, fibroblasts and VSMCs (Lazarus *et al.*, 1995; Li *et al.*, 2000; Nelson *et al.*, 1988). LOX has been associated with various pathologies, including cardiovascular diseases (Rodriguez *et al.*, 2008), neurodegenerative disorders (Gilad *et al.*, 2001; Gilad *et al.*, 2005), tumor progression and metastasis (Erler *et al.*, 2006; Min *et al.*, 2009; Payne *et al.*, 2005). An interesting insight into the regulation of MKs by LOX came from a recent study from our laboratory showing that LOX can oxidize cell surface proteins, including PDGFR-α, in rat aortic smooth muscle cells (Lucero *et al.*, 2008). This effect was blocked by α-aminopropionitrile (BAPN), an inhibitor of LOX enzyme activity (Lucero *et al.*, 2008; Tang *et al.*, 1983). The BAPN-mediated inhibition of PDGFR oxidation diminished the binding affinity for its correspondent ligand, PDGF-BB. This inhibition resulted in an accelerated rate of decay of phosphorylated downstream effectors of PDGFR signaling, such

as Akt and ERK1/2 (Lucero *et al.*, 2008). This effect of LOX was also identified in MKs, showing dependency of PDGF-BB on an active LOX to promote MK proliferation. Furthermore, LOX is primarily expressed in low-ploidy MKs (Eliades *et al.*, 2011).

Our laboratory also uncovered an important role for LOX in controlling MK-induced bone marrow fibrosis. The term myelofibrosis indicates BM deposition of reticulin, collagen or both. However, regardless of the pathogenic background, MK-induced myelofibrotic conditions share a common denominator: defective MK development and a dense extracellular matrix (Kuter *et al.*, 2007). BM fibrosis, in the context of acute megakaryocytic leukemia (AMKL) and myeloproliferative disorders usually involves deposition of reticulin and/or collagen fibers (Kuter *et al.*, 2007; McCarthy, 1985). The evolving hypothesis is that MKs release growth factors such as Transforming Growth Factor β (TGF-β), PDGF and Fibroblast Growth Factor (FGF), which accentuate the production of collagen by fibrogenic cells (Le Bousse-Kerdiles and Martyre, 1999; Le Bousse-Kerdiles *et al.*, 2008; Reilly *et al.*, 1993; Terui *et al.*, 1990; Vannucchi *et al.*, 2002). LOX not only affects the proliferative effect of PDGF, but through its ability to cross-link the extracellular matrix, is a major regulator of the fibrotic phenotype in the above pathologies. Pharmacological inhibition of LOX significantly attenuated matrix deposition and myelofibrosis in GATA-1^{low} mice (Eliades *et al.*, 2011). In GATA-1^{low} mice, the abrogation of the distal promoter of GATA-1 and the DNAse hypersensitive region leads to downregulated GATA-1. These mice display myelofibrosis with a significantly increased number of MKs arrested between the stage of megakaryoblast and immature MK and decrease in total marrow cellularity (Centurione *et al.*, 2004; Vannucchi *et al.*, 2002). The effects of LOX on MKs are summarized in Figure 3.

Finally, a study analyzing the levels of oxidative stress in patients with primary myelofibrosis detected significantly raised ROS concentrations and significantly lowered total antioxidant capacity (Vener *et al.*, 2010). Added to the finding that ROS were implicated in the expression of hypoxia-induced LOX in endothelial cells (Guadall *et al.*, 2011), the data accumulated so far suggest a novel and central role of ROS and LOX in the progression of marrow fibrotic disorders.

Therapeutic targeting of ROS

ROS homeostasis deregulation has been reported in many hematological disorders. Together with evidence of ROS involvement in regulation of critical cellular events such as proliferation, differentiation and survival, targeting ROS for therapeutic purposes is a promising approach for further development. However, since ROS are also important for homeostasis of normal hematopoietic cells, specific targeting of malignant cells has proven trickier than initially hoped. Two basic approaches are possible: the pro-oxidant and anti-oxidant, which aim respectively to increase or decrease ROS in target cells (Grek *et al.*, 2011; Hole *et al.*, 2011; Sardina *et al.*, 2011).

The rationale of the pro-oxidant approach is that since malignant cells already have high levels of ROS, increasing their ROS to toxic levels is more easily achievable than with normal cells. This effect may be achievable by inhibiting intracellular anti-oxidants. TLK 199, *Telintra*, is a peptido-mimetic inhibitor of an isoform of GST, GSTP (π). In preclinical mouse studies *Telintra* raised circulation of blood cells of all lineages, an effect associated with increase in BM progenitor cells. *Telintra* has shown positive results in an ongoing Phase II clinical trial for MDS; multilineage hematologic improvement has been observed, including decreased requirements for red blood cell, platelet, and growth factor support (Grek *et al.*, 2011).

The anti-oxidant approach is based on the idea that malignant cells depend on high levels of ROS for survival. Amifostine has been used with traditional anti-cancer drugs as a

cytoprotective agent. Amifostine is converted to its active metabolite by the membrane-bound enzyme alkaline phosphatase. The active metabolite prevents or repairs oxidative stress-induced DNA damage by scavenging free radicals, donating hydrogen ions to free radicals and direct binding and inactivation of cytotoxic drugs. Because normal tissues generally have higher levels of alkaline phosphatase, better vascularization and higher pH, the active metabolite preferentially locates there. Studies have also indicated that amifostine stimulates HSCs. These features have inspired ongoing clinical use of amifostine in MDS patients (Grek *et al.*, 2011; Hole *et al.*, 2011).

Summary

There is increasing evidence that oxygen tension in the microenvironment affects MK differentiation, maturation, polyploidy and proplatelet fragmentation. Further research is needed to better understand how different oxygen tensions translate into signals that control MK biology. As outlined above, an array of oxidases, including NOX or LOX, affect the bone marrow niche and its matrix, as well as cell cycle properties. Together, these influences have a major impact on lineage development and cell propagation. Developing selective inducers or inhibitors of specific oxidases to control ROS in the BM niche will aid investigations at both the basic and translational levels.

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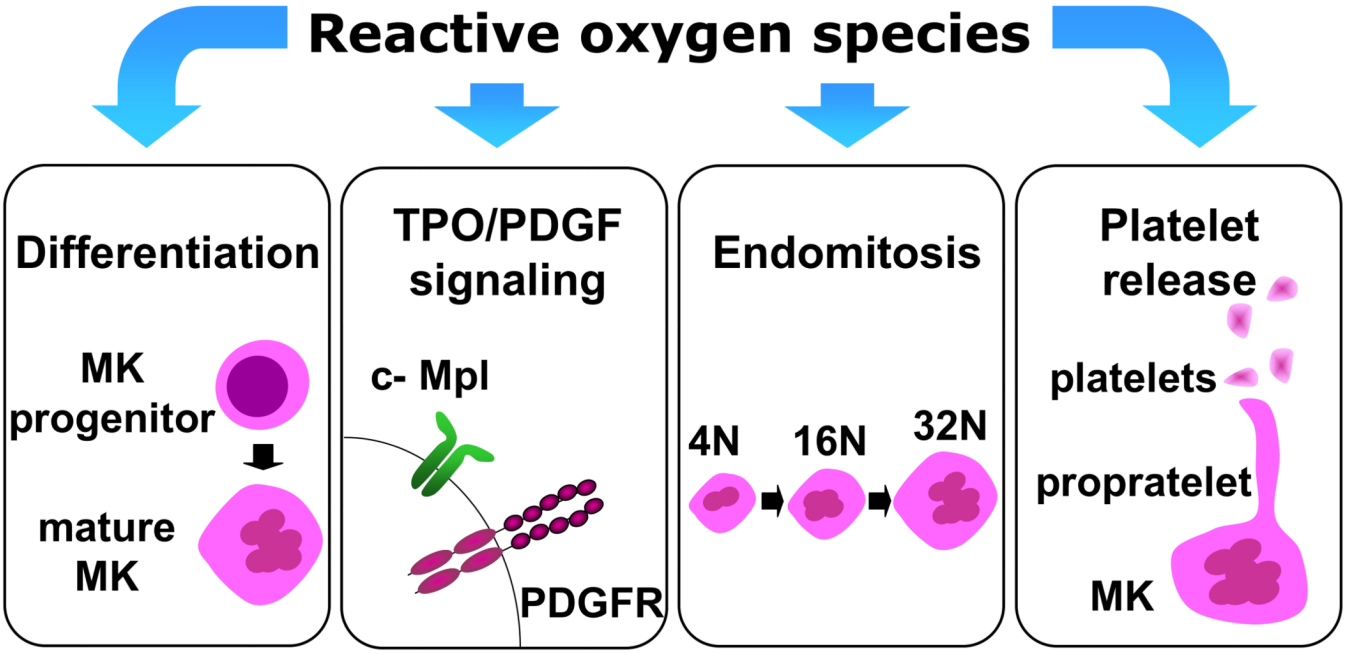

Figure 1. Effects of ROS on MK biology

Schematic illustration of the major reported effects of ROS on MK biology, covered in this review. MK: megakaryocyte; TPO: thrombopoietin; c-Mpl: thrombopoietin receptor; PDGF: platelet-derived growth factor; PDGFR: PDGF receptor.

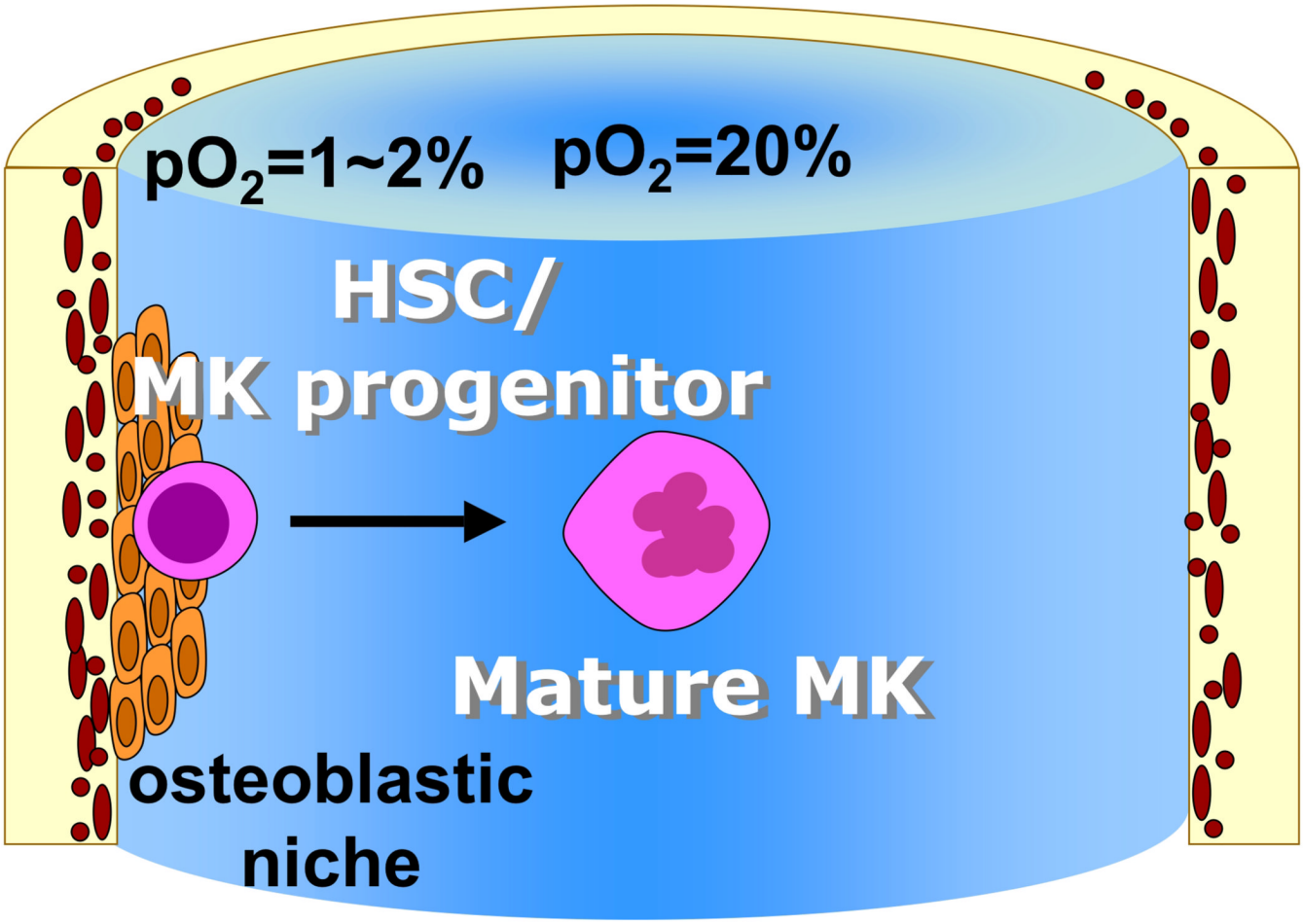

Figure 2. Spatial organization of the BM, with respect to distribution of oxygen tension and its effect on MK differentiation

pO2: oxygen tension; HSC: hematopoietic stem cell; MK: megakaryocyte.

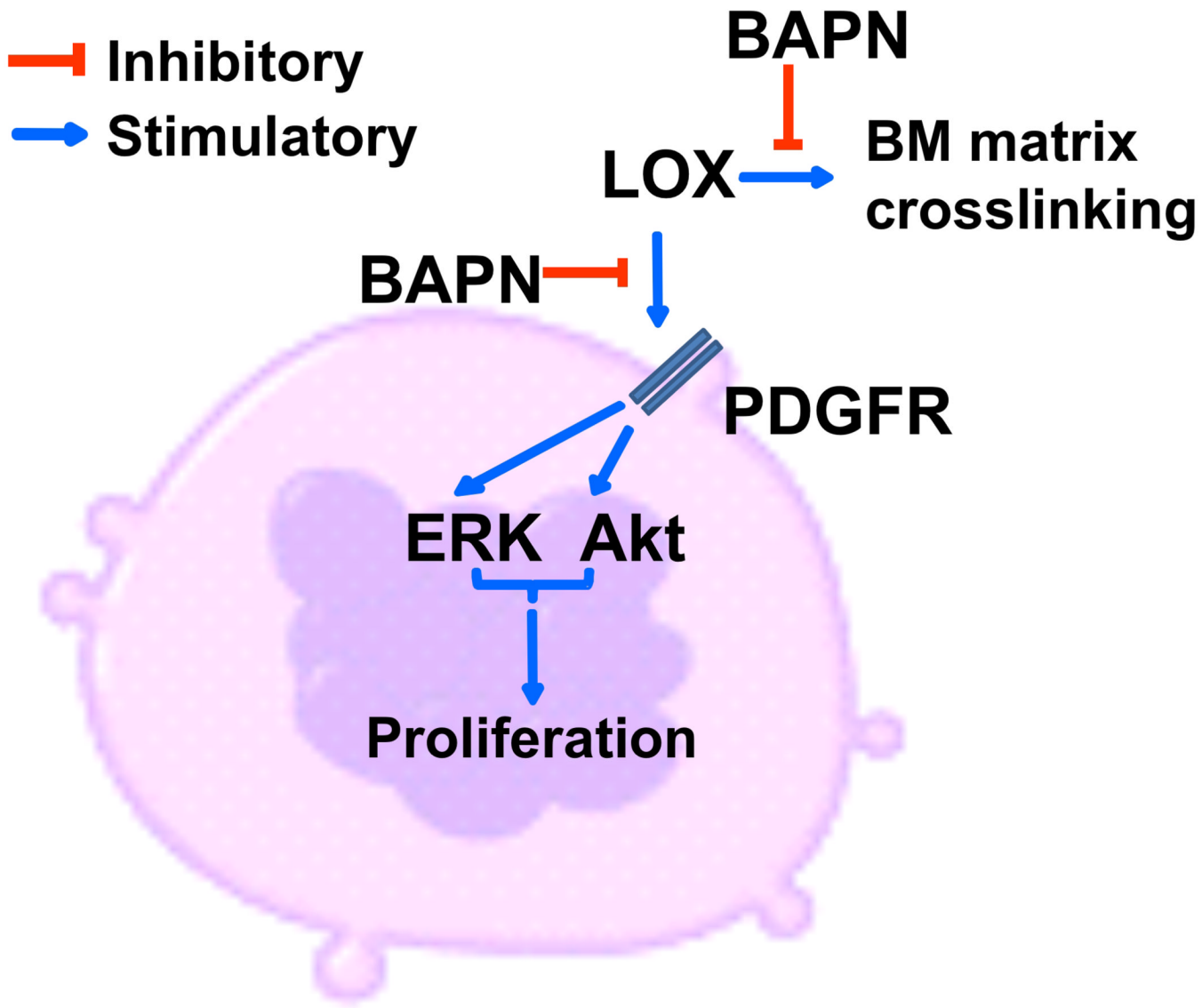

Figure 3. Effect of LOX on MK biology and progression of BM fibrosis

Extracellular LOX stimulates PDGF-mediated Erk and Akt signaling, contributing to MK progenitor proliferation. Inhibition of LOX by BAPN eliminates this effect. BAPN also inhibits collagen cross-linking through inhibition of the catalytic activity of LOX, hampering progression of myelofibrosis.