

Review

ROS-mediated platelet generation: a microenvironment-dependent manner for megakaryocyte proliferation, differentiation, and maturation

S Chen¹, Y Su¹ and J Wang^{*,1}

Platelets have an important role in the body because of their manifold functions in haemostasis, thrombosis, and inflammation. Platelets are produced by megakaryocytes (MKs) that are differentiated from haematopoietic stem cells via several consecutive stages, including MK lineage commitment, MK progenitor proliferation, MK differentiation and maturation, cell apoptosis, and platelet release. During differentiation, the cells migrate from the osteoblastic niche to the vascular niche in the bone marrow, which is accompanied by reactive oxygen species (ROS)-dependent oxidation state changes in the microenvironment, suggesting that ROS can distinctly influence platelet generation and function in a microenvironment-dependent manner. The objective of this review is to reveal the role of ROS in regulating MK proliferation, differentiation, maturation, and platelet activation, thereby providing new insight into the mechanism of platelet generation, which may lead to the development of new therapeutic agents for thrombocytopenia and/or thrombosis.

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Facts

- Platelet production is a consecutive process beginning with haematopoietic stem cell (HSC) proliferation and ending with the fragmentation of megakaryocytes (MKs). This process occurs in a microenvironment called the 'niche'.
- Reactive oxygen species (ROS) affect the fate of HSCs by regulating the ROS-phosphatidylinositol-3-kinase (PI3K)/Akt-transcription factor forkhead box O (FOXO)-3 and ROS-p38 mitogen-activated protein kinase α (MAPK α) pathways.
- P22^{phox}-dependent nicotinamide-adenine dinucleotide phosphate oxidase (NOX)-1 may be responsible for ROS generation in the regulation of MK commitment and differentiation.
- Platelet production is a result of MK apoptosis, which may be caused by elevated levels of ROS.

Open Questions

- Which NOX isoform is responsible for the different stages of thrombocytopoiesis, including MK proliferation, differentiation, maturation, and fragmentation?
- Where is the boundary between the physiological and pathophysiological effects of ROS on MK differentiation and platelet activation?
- What is the relationship between ROS and cytokine-receptor signalling during MK differentiation?

Approximately 1×10^{11} platelets are produced by MKs every day in the human body.^{1,2} The best-known function of platelets is their response to blood vessel injury, in which they change shape, secrete granule contents, and aggregate to prevent blood loss. In addition, studies have shown that platelets also have an important role in several other

¹College of Preventive Medicine, State Key Laboratory of Trauma and Burns and Combined Injury, Institute of Combined Injury of PLA, Chongqing Engineering Research Center for Nanomedicine, College of Preventive Medicine, Third Military Medical University, Chongqing 400038, People's Republic of China

*Corresponding author: J Wang, College of Preventive Medicine, State Key Laboratory of Trauma and Burns and Combined Injury, Institute of Combined Injury of PLA, Third Military Medical University Chongqing, Chongqing 400038, China. Tel: + 86 23 68752283; Fax: + 86 23 68752283;

E-mail: wangjunping@tmmu.edu.cn or wangjunp@yahoo.com

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Abbreviations: AP-1, activation protein-1; ATO, arsenic trioxide; BM, bone marrow; CNC, Cap'N'Collar; DPI, diphenyliodonium; ERK, extracellular signal-regulated kinase; FOXO, transcription factor forkhead box O; GM-CSF, granulocyte-macrophage colony-stimulating factor; H₂O₂, hydrogen peroxide; HSCs, haematopoietic stem cells; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MK, megakaryocyte; NAC, N-acetylcysteine; NF- κ B, nuclear factor- κ B; NO, nitric oxide; NOX, nicotinamide-adenine dinucleotide phosphate oxidase; PHOX, phagocytic oxidases; PI3K, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; SDF-1, stromal cell-derived factor-1; STAT, signal transducers and activators of transcription; TLR, Toll-like receptor; TPO, thrombopoietin; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂

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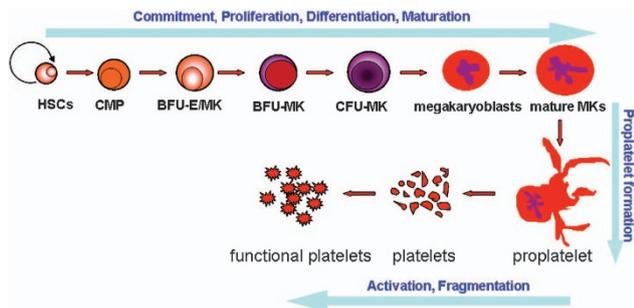


Figure 1 Overview of all biological phases of platelet generation from HSCs and subsequent platelet activation. The abbreviated cell types are: CMP, common myeloid progenitor; BFU-E/MK, burst-forming unit erythrocyte/megakaryocyte; BFU-MK, burst-forming unit megakaryocyte; CFU-MK, colony-forming unit megakaryocyte

processes, such as inflammation, neoangiogenesis, innate immunity, and tumour metastasis.^{3–6} Platelets are produced during several stages involving the commitment of HSCs to the MK lineage, MK progenitor proliferation, MK differentiation and maturation, and platelet release from mature MKs as a consequence of apoptosis^{7,8} (Figure 1). As reported previously, thrombocytopoiesis is regulated by a variety of haematopoietic cytokines, such as thrombopoietin (TPO), granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin family proteins (IL-1, IL-3, IL-6, and IL-11), and stromal cell-derived factor-1 (SDF-1).^{9–13} However, the mechanisms underlying MK differentiation and platelet generation under physiological and pathological conditions have not been adequately elucidated.

Research over the past 20 years has demonstrated that reactive oxygen species (ROS) have an essential role in certain cellular events.¹⁴ Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases (NOXs) is regarded as an important intercellular producer of ROS.¹⁵ However, beside NOXs, ROS may be generated as by-products, such as superoxide anion leaked from mitochondrial respiratory chain, hydrogen peroxide (H_2O_2) generated by phagocytic oxidases (PHOX) in the plasma membrane, and hypochlorous acid catalyzed by myeloperoxidase in activated neutrophils.^{16,17} In addition, other enzyme systems, including xanthine oxidoreductase, cyclooxygenase, uncoupled nitric oxide (NO) synthases, P450 mono-oxygenase, and lipoxygenase, have also been identified as potential ROS donors.^{18–20} ROS can affect pathogen defences, post-translational protein processing, gene expression regulation, and cell signalling and differentiation.¹⁵ Recently, studies have suggested that ROS are also involved in both MK development and platelet activation. The objective of this article is to review and identify the properties and functions of ROS in regulating MK differentiation and maturation, and platelet production and activation. We believe that this article will provide additional information regarding megakaryocytopoiesis and platelet generation.

Oxidation State of the Bone Marrow Microenvironment: An Important Determinant for HSC Fate

In 1978, Schofield²¹ proposed the HSC ‘niche’ model for the physiological microenvironment of HSCs. Later, studies

provided evidence that niche is just a site composed of heterologous cell types that created three-dimensional structure for stem cell residing.²² Recently, a new definition for niche is proposed as a composition of two stem cells: HSCs and osteoprogenitor, with the latter contributing to the cavity formation, sinusoid development, and osteoblast and adipocyte production, thereby embodying different functional cues for HSCs in turn.²³ So far, it is widely recognised that niche is a regulatory system that controls HSCs’ activity from their surroundings, including supporting cells and their secreted factors, nervous system, and oxygen, and so on.^{24–26} In recent years, increasing studies of the niche have focused on its important role in determining stem cell fate with regard to self-renewal *versus* differentiation.^{22,27–29} Two components of the niche have been identified for the different HSC activity states: the osteoblastic niche and the vascular niche. The osteoblast lining of the endosteal surface is an important component of the osteoblastic niche, and the adhesion of HSCs to osteoblasts is a critical molecular mechanism for maintaining quiescent HSCs.^{30,31} Haematopoietic cells and endothelial cells are the two elements of the vascular niche, which is the site for the proliferation and differentiation or maturation of HSCs into circulation.^{32–34}

HSC distribution in the niche is closely associated with O_2 tension.³⁵ The endosteal surface of the niche has the lowest O_2 level,³⁶ where primitive HSCs are maintained in the quiescent state (G_0). It is known that 0.1% O_2 prevents $CD34^+$ cells from entering into the cell cycle.³⁷ Furthermore, hypoxia augments the colony-forming potential of human haematopoietic progenitor cells and their potential to differentiate into MKs, because 1% O_2 could not only promote the proliferation of $CD34^+$ cells but also increase the expression of $CD41$ ³⁸ (Figure 2). In addition, altered superoxide within stromal cells might lead to abnormal survival and development of haematopoietic cells in the myelodysplastic marrow microenvironment,³⁹ and higher levels of H_2O_2 secreted from stromal cells in the bone marrow (BM) could be detected with lower peroxiredoxin 2 expression in the haematopoietic niche,⁴⁰ further suggesting that the niche can regulate the activity of HSCs in an ROS-dependant manner.

HSCs also modulate several molecules to adapt to the hypoxic environment, such as glycolytic and oxidative repair proteins, which protect them from oxygen radicals.⁴¹ Furthermore, HSCs can regulate the level of hypoxia-inducible factor-1 α ,⁴² accompanied by the increased expression of Tie-1 and vascular endothelial growth factor, which adjusts the O_2 gradient to promote HSC homing to the niche.^{43,44} Specifically, HSC/progenitor cell compartment showed an approximately 27-fold expansion under a 5% O_2 atmosphere. However, less expansion and cell cycle activation suppression were observed under normoxic conditions.⁴⁵ Because it is widely believed that oxidative metabolism generates ROS, these results indicate that low levels of ROS may contribute to HSC quiescence and self-renewal, whereas the gradual increase of ROS is associated with HSC expansion. However, an increase in ROS beyond a certain level will harm HSCs because of toxic effects.

Several studies have suggested that ROS may participate in or enhance the intracellular signalling of HSCs in either a quiescent or proliferative state. Mdm2, a gene coding for an

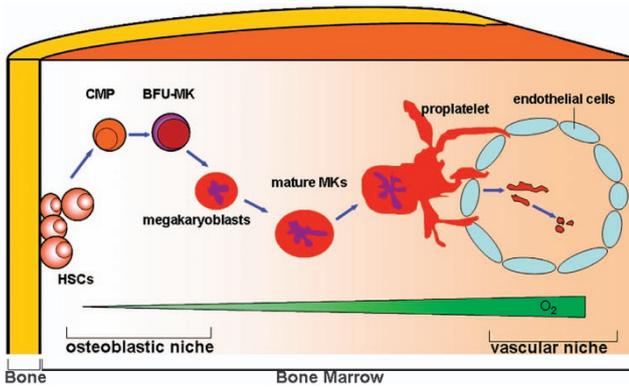


Figure 2 The distribution of MKs in their niche. MK commitment from HSCs and differentiation into immature MKs occur in the osteoblastic niche. Proplatelets then adhere to the vasculature and ultimately release individual platelets from their tips after mature MKs migrate to the vascular niche. The abbreviated cell types are: CMP, common myeloid progenitor; BFU-MK, burst-forming unit megakaryocyte

E3 ubiquitin ligase,⁴⁶ Abbas *et al.*⁴⁷ reported that basal ROS accumulated and p53 activity increased during HSC development when Mdm2 was knocked out, and p53 could further increase ROS production by transactivating p53-induced genes (PIG). As *Mdm2* has the ability to control p53 in a low level, which is critical for cellular homeostasis and allows for stem cell survival,⁴⁷ it is taken as an important factor to regulate ROS levels during normal haematopoiesis. ROS overaccumulation can also be prevented by nuclear factor- κ B (NF- κ B) and the antioxidant activity of NF- κ B is considered to be indispensable for the development of haematopoietic progenitor cells, as Flk-1⁺ cells (putative haemangioblasts) expressing dominant-negative Rel/NF- κ B (*I κ BSR*) exhibited higher apoptosis through increasing ROS accumulation.⁴⁸

The FOXO is also required for the quiescence and homeostasis of HSCs because it maintains a low threshold of intracellular ROS levels.^{49–51} Notably, activated AKT (protein kinase B) directly phosphorylated FOXO, leading to increased ROS production and FOXO-3 mobilization to the nucleus when ROS accumulated. This was accompanied by cell proliferation.⁵⁰ Elevated ROS levels also induce HSC-specific p38 MAPK α phosphorylation, followed by defects in HSC maintenance,⁵² whereas activated p38 MAPK α further promotes ROS production to expand CD133⁺ cells.⁵³ These results suggest that PI3K/Akt-FOXO-3 signalling and p38 MAPK α pathway have an important role in the ROS-dependent regulation of HSC expansion. In addition, ROS also appear to affect other functions of HSCs because murine Lin⁻c-Kit⁺ cells deficient in NOX2 showed impaired chemotaxis, invasion, and actin reorganisation when exposed to SDF-1,⁵⁴ whereas *N*-acetylcysteine (NAC) (an ROS scavenger) treatment suppressed the mobilisation of HSCs.⁵⁵

Besides ROS, the thiol balance is also reported to contribute to the myeloproliferation and haematopoietic progenitor cell mobilisation, as redox status can be regulated by free or protein-incorporated thiols.⁵⁶ Interestingly, a shift in the thiol–disulphide equilibrium sustained by the glutathione/thioredoxin will occur in early steps during differentiation, which makes the committing cells more proficient to handle an increased level of ROS.⁵⁷

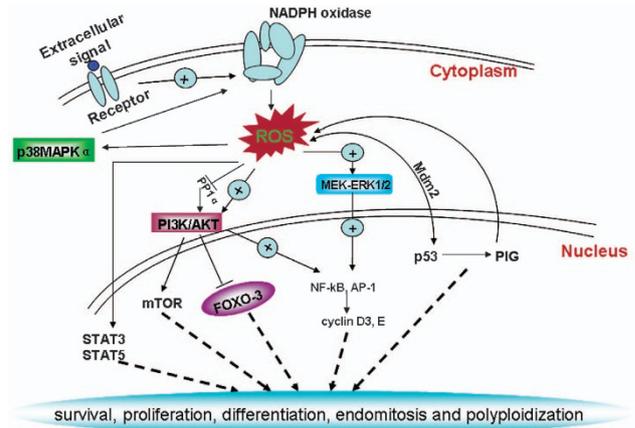


Figure 3 Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase-dependent ROS production is required for all phases of megakaryocytopoiesis. ROS production or augmentation is stimulated by extracellular signals, such as cytokines, endogenous ROS, and other ligands. ROS is necessary for the complete activation of signalling pathways as a second messenger in the regulation of signal transduction during MK development, and ROS might be the central player during megakaryocytopoiesis

In conclusion, HSCs are maintained in a quiescent state for self-renewal in the osteoblastic niche with low levels of ROS via the regulation of p53 levels, NF- κ B activity, and with thiol balance. Whereas in a more oxygenated environment, HSCs and other progenitor cells actively proliferate, migrate, and differentiate. This is partly due to the effect of ROS on HSC development through the regulation of the ROS-PI3K/Akt-FOXO-3 and ROS-p38 MAPK α pathways, and the shift of thiol–disulphide equilibrium (Figure 3).

Significance of ROS in the Lineage Commitment of HSCs to MKs

HSCs proliferate and differentiate into all haematopoietic lineages after they lose their self-renewal capacity in response to various extracellular or intracellular agonists. Specifically, ROS act on the differentiation phase of HSCs into MKs, as addition of the antioxidant 6-hydroxy-2,5,7,8-1 tetramethylchroman-2-carboxylic acid (Trolox) and NAC inhibited the acquisition of MK morphological features and significantly lowered the levels of the MK markers CD41, CD42b, and CD61 induced by TPO.⁵⁸ In addition, 5% O₂ increased the total number of MKs and CFU-MKs from CD34⁺ cells.⁵⁹ Sardina *et al.*⁵⁸ observed that p22^{phox} NOX-dependent complexes had an important role in regulating ROS production during megakaryocytopoiesis. NOX1 may be the most abundant NOX isoform in MKs and may be upregulated by TPO, and TPO-stimulated ROS levels can be abrogated by an NOX inhibitor, diphenyliodonium (DPI).⁶⁰

However, the mechanism through which ROS activate the signalling cascades required for MK commitment remains unclear. Previous studies have suggested that exogenous ROS could increase the tyrosine phosphorylation of the common β c and signal transducers and activators of transcription-5 (STAT5). Moreover, the tyrosine residues of other signalling molecules, including c-KIT, the adapter protein SHC, and the protein tyrosine phosphatase SHP-1,

were also phosphorylated after H₂O₂ stimulation in the MK cell line MO7e.⁶¹ These results indicate that a change in the redox state increases the tyrosine phosphorylation of cellular proteins. The effect of ROS on phosphatase inhibition, which regulates these signalling pathways, may be a significant mechanism of MK commitment because PP1 α inhibition by ROS constitutively activates the PI3K/AKT pathway in leukaemic cells.⁶²

The effect of ROS on the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)-extracellular signal-regulated kinase (ERK) 1/2 pathway may be another possible mechanism underlying MK commitment. ERK activation in K562 and HEL cells induced by phorbol myristate acetate during MK differentiation was also inhibited by ROS inhibitors.⁵⁸ Furthermore, MEK-ERK1/2 signalling was shown to have a critical role in MK commitment and differentiation because inhibition of the MEK-ERK1/2 pathway with two structurally distinct MEK inhibitors (UO126 and PD186141) increased the expression of the immature marker CD34⁺ and decreased the expression of the late MK commitment marker GPIb (CD42b).⁶³ In addition, inhibition of the MEK-ERK1/2 pathway caused a leftward shift of the ploidy in BM and foetal liver-derived primary MKs, showing a critical requirement for the MEK-ERK1/2 pathway in MK differentiation from HSCs.⁶³ These results indicate that ROS may be a vital regulator that acts on different signalling pathways during megakaryocytic development. Regrettably, the details of the mechanism of ROS in regulating cellular signalling pathways during megakaryocytic development have not been fully uncovered.

The Role of ROS in Regulating MK Proliferation and Differentiation

Recent studies have shown that ROS promote the proliferation of many cell types.^{64–68} Moreover, ROS are increased in a cell cycle-dependent manner and oscillated with every cell division, indicating that ROS may also affect cell cycle progression.⁶⁹ Notably, it has been shown that MK differentiation from HSCs occurs in the osteoblastic niche, which is partially attributed to the close relationship between ROS and MKs⁷⁰ (Figure 3).

Stimulating human haematopoietic cells with GM-CSF, IL-3, stem cell factor, or TPO, which act on different stages of megakaryocytopoiesis,^{71,72} will trigger ROS production. High H₂O₂ concentrations can increase intracellular ROS levels, which is accompanied by several events, including G1- to S-phase cell cycle progression, c-fos gene expression, and cell migration. Surprisingly, the ROS levels and signal activation were decreased when cells were treated with the antioxidant pyrrolidine dithiocarbamate, indicating a role of ROS signalling in MK proliferation.⁶¹

Specifically, ROS are upstream of the signalling cascades required for MK proliferation and differentiation, because DPI could inhibit the activation of AKT, STAT3, and STAT5, which are hypothesised to be necessary for MK differentiation.⁵⁸ Furthermore, in p22^{phox} knocked-down cells, ERK, STAT-5, and AKT activations were also significantly lower.⁵⁸ ROS also regulate the activation or induction of transcription factors, including p53, NF- κ B, and activation protein-1 (AP-1). The family of JUN/FOS transcription factors and the regulation of

AP-1 by redox processes show that ROS also mediate the function of AP-1 in MK growth and proliferation.⁷³ Furthermore, glycolysis activation is a critical step for ROS production because the glucose transport rate is one of the first responses following ligand–receptor interactions,⁷⁴ and ROS production is tightly coupled with glucose metabolism.⁷⁵ However, inhibition of glucose utilisation could decrease ROS production induced by TPO in a PI-3K/AKT-dependent manner in UT-7/TPO cells.⁷⁶

During the unique endomitosis (a specific phenomenon characterised with abortion of mitosis in late anaphase and failure of cytokinesis followed by reinitiation of DNA synthesis) and polyploidisation processes of MK development, the cell size induced by TPO was greater under higher O₂ tension conditions,⁷⁷ and the MKs also showed a higher ploidy under high O₂ tension conditions.⁵⁹ This suggests that endomitosis and polyploidisation are closely related to ROS levels. Specifically, in endomitosis and polyploidisation development, ROS may mediate the progression of G1–S phase by regulating G1 cyclin gene expression,⁷⁸ and NOX inhibitors can downregulate the effects of cyclins D3 and E on MK polyploidisation.⁷⁹

As reported, cyclin D3 is the predominant D-type cyclin because it can induce high ploidy levels in cyclin D3 transgenic mice and the ablation of cyclin D3 in cultured MKs can significantly decrease MK ploidy.^{80,81} Cyclin E is also a G1-phase cell cycle regulator and has been suggested to have a role in MK ploidy promotion, which has been shown in megakaryoblastic K562 cell re-replication cycles.⁸² The ploidy levels in NOX-inhibited MKs were partially restored when cyclin E was upregulated, which further supports the involvement of cyclin E in MK polyploidisation through the G1/S-phase regulation.⁶⁰ However, NOX1-deficient mice displayed no differences in MK ploidy compared with wild-type mice, whereas NOX inhibitors could cause a significant decrease in the ploidy of MKs. This indicates that NOX1 alone is not necessary for MK polyploidisation.⁶⁰

Role of ROS in MK Maturation and Platelet Release

Mature MKs are in proximity to the BM sinusoid space or lung capillaries, where there is a high level of O₂, and that MKs can release platelets into the sinus in this region.⁸³ An increase in either O₂ tension or ROS promotes the maturation of MKs.⁸⁴ Early studies of thrombopoiesis showed that CD34⁺ peripheral blood cells generated more CD41⁺ MKs under 20% pO₂ than under 5% pO₂ conditions⁵⁹ and that MK differentiation under 20% pO₂ was associated with increased expression of the MK maturation-specific transcription factors GATA-1 and NF-E2.⁸⁵ Of the two transcription factors, GATA-1 was shown to interact with either RUNX1 or FOG-1 to promote MK differentiation and affect cytoplasmic maturation before platelet release.⁸⁶ NF-E2 may coordinate with other genes that reorganise the cytoskeleton and transport organelles into proplatelets to regulate platelet release because NF-E2-null mice did not undergo proplatelet formation.⁸⁷ Therefore, it is hypothesised that MK maturation is closely related to the cellular response to oxidative stress.

However, platelet generation is also associated with NO, which is constitutively produced by endothelial cells. Reports

have shown that exogenous and endogenous forms of NO induce apoptosis in MKs.⁸⁸ Battinelli *et al.*⁸⁹ observed that, in the human megakaryocytoid cell line Meg-01, NO-induced apoptosis was associated with the terminal stages of megakaryocytopoiesis, including proplatelet formation, platelet release, and, ultimately, MK death. Notably, studies indicated that NO was easily inactivated by O₂ to form another type of ROS, ONOO⁻.⁹⁰ ONOO⁻ activates upstream effectors of ERK, such as MAPK/ERK,⁹¹ which substantially increases thrombopoiesis during the last phase of MK development. Therefore, NO may interact with O₂ to form ROS, which act during the final stage of MK maturation in an ERK-dependent manner. This may be a possible explanation for the effect of ROS on MK maturation and platelet release (Figure 3).

In addition, there appears to be an inner transcriptional mechanism through which ROS accumulation promotes MK maturation. Nrf2, a Cap'N'Collar (CNC) transcription factor, confers cytoprotection against oxidative stress through heterodimerisation with small Maf proteins.^{92–94} It was reported that the expressions of Nrf2-targeted genes involved in the stress response were decreased during MK maturation. This suggests that MKs may reduce the concentration of antioxidant proteins during maturation, which facilitates MK development by using ROS accumulation as a maturation signal.⁹⁵ Another CNC transcription factor, p45, which is a component of NF-E2,⁹⁶ has a critical role in megakaryocytic maturation and platelet production,⁹⁷ as proplatelet formation was completely defective in p45-null MKs.⁸⁷ A recent study indicated that the increase in p45 could promote ROS accumulation and full activation of the platelet genes that promote thrombocytopoiesis.⁹⁸

In addition, studies have shown that ROS generation is increased during the terminal phase of platelet production. For example, the transcription factors associated with MK maturation, GATA-1, NF-E2, and mammalian target of rapamycin, were all increased and that this increase was accompanied by increased ROS production when Toll-like receptor-2 (TLR2) activated, which is reported to express on MKs.^{99,100} Therefore, it is postulated that TLR2 drives MK maturation through ROS production. Another transcription factor, peroxisome proliferator-activated receptor γ , is also found to be present in both MKs and platelets,¹⁰¹ and the treatment with its ligand, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), may enhance the proplatelet formation.⁸⁴ Further investigation revealed that the effect of 15d-PGJ₂ was associated with the modulation of intracellular ROS levels in MKs, because 15d-PGJ₂-induced platelet production was partially blocked with antioxidants.⁸⁴ Moreover, the observation that reduced MK apoptosis and platelet production were accompanied by a decrease in total ROS levels in the cells suggested that ROS were also involved in MK apoptosis to generate platelets.¹⁰² Taken together, these results suggest that ROS had an important role in MK maturation and/or subsequent proplatelet formation.

Close Correlation between ROS and Platelet Activation

Platelets that circulate in the bloodstream under normal conditions will actively adhere to and spread on the collagen matrix if the vascular endothelial layer is injured. ROS are also

involved in this process as well as platelet aggregation and recruitment, because blocking superoxide production could suppress platelet activation and aggregation.^{103–105} However, there remains some controversy regarding the mechanism by which oxidants affect platelet function.¹⁰⁶

Collagen-induced platelet aggregation was associated with H₂O₂ release, which acts as a second messenger by stimulating the arachidonic acid metabolism and phospholipase C pathways,^{107,108} suggesting that there are potential sources of ROS in platelets themselves. Further studies suggested that platelet aggregation may be partially mediated by the NOX system and phospholipase A2-activated arachidonic acid metabolism, and protein kinase C may modulate both pathways.¹⁰⁹ The nicotinamide adenine dinucleotide oxidase Ecto-NOX1 has been described in human platelets, and ROS production triggered by ligand–receptor interactions could thereby enhance the expression and activity of Ecto-NOX1. Therefore, it is hypothesised that redox regulation of Ecto-NOX1 may have a significant role in platelet activation and recruitment.¹¹⁰

However, it is unclear how ROS function within platelets. Earlier studies demonstrated that rapid platelet activation was induced by the acute inhibition of endogenous NO production *in vivo*,¹¹¹ whereas ROS may partially modulate platelet function by attenuating NO bioavailability via ONOO⁻ generation.¹¹² Recent studies indicated that ONOO⁻ can react with tyrosyl residues to form 3-NO₂-Tyr and with thiols to generate S-nitrosothiols, which reduces the inhibitory effect of NO. In addition, ONOO⁻ may act as a substrate for COX peroxidase, leading to prostaglandin endoperoxide H synthase activation and increased prostaglandin formation.¹¹³ These reactions are important for platelet-derived thromboxane A₂ synthesis.¹¹⁴ Moreover, platelet-derived ONOO⁻ can lead to the production of 8-iso-PGF_{2a} from arachidonic acid,¹¹⁵ whereas 8-iso-PGF_{2a} may increase calcium release from intracellular stores, induce platelet shape change, and amplify platelet aggregation in response to agonists.¹¹³ Furthermore, ONOO⁻ is likely to enhance the bioavailability of adenosine diphosphate, thereby increasing additional platelet recruitment.¹¹⁵

ROS production affects α IIb β 3 activation because NOX inhibitors and superoxide scavengers may reduce platelet aggregation and α IIb β 3 activation.¹¹⁶ In addition, ROS could also increase platelet adhesion to the vascular endothelium by acting on transcriptional mechanisms in the endothelium, degrading the endothelial glycocalyx, and inactivating endothelial ectonucleotidases.¹¹⁷ It is hypothesised that platelet agonists, such as thrombin, collagen, and thromboxane, can activate NOX, thereby causing intracellular ROS accumulation and affecting several phases of platelet activation (Figure 4). In turn, platelets may release superoxide anions into the bloodstream as autocrine messengers, which will be involved in further platelet activation and recruitment to thrombus formation sites.¹¹⁵

ROS-Related Therapeutic Strategies for Thrombocytopenia and Thrombosis

Because ROS can influence the function and activity of various molecules involved in the commitment, proliferation, differentiation of MKs, and platelet release, as well as platelet

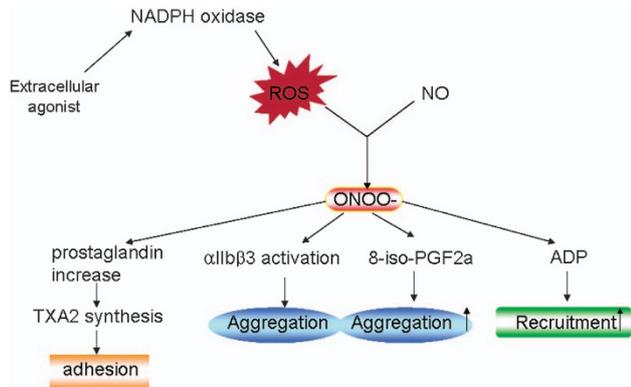


Figure 4 Proposed ROS signalling cascades and cellular biological outcomes after ROS interact with NO to generate ONOO⁻

activation and aggregation (Figure 4), this characteristic of ROS makes them interesting therapeutic targets for regulating megakaryocytopoiesis and platelet function. Indeed, modulating ROS levels has previously been shown to be a promising strategy for treating the abnormal decrease or increase in platelet count under pathological conditions. The two opposing strategies for treating abnormal platelet numbers are to either decrease or increase ROS levels accordingly.

As reviewed, the gradual rise in ROS levels is tightly related to MK development. Therefore, increasing the level of ROS either through the enhancement of ROS generation or inhibition of intracellular antioxidants will provide potential therapeutic avenues for rescuing thrombocytopenia. For instance, the ability of 15d-PGJ₂ to increase MK ploidy and proplatelet formation and to promote platelet recovery after ionising radiation is primarily attributed to its capacity to induce ROS formation.⁸⁴ Further investigation revealed that inhibiting haem oxygenase-1 activity could enhance 15d-PGJ₂-induced platelet production.¹¹⁸ Moreover, arsenic trioxide (ATO), a miracle drug for the treatment of relapsed acute promyelocytic leukaemia, also displayed a haemostatic effect, probably due to the fact that ATO could enhance either mitochondrial ROS generation or NOX activation through the inhibition of thioredoxin and increase the permeability of the mitochondrial membrane.^{119,120} It is known that currently, as the main treatment for thrombocytopenia, platelet transfusion is expensive, time-consuming, risky, and has limited efficacy (alloimmunisation).¹²¹ Hence, it is desirable to develop ROS-dependent agents for the treatment of thrombocytopenia.

An abnormal increase in platelets and activation leads to other types of diseases, such as thrombosis and atherosclerosis,^{122–124} which are accompanied by increased intraplatelet ROS production.^{125–128} Therefore, decreasing ROS levels may be an effective strategy for treating these types of diseases. Recently, several agents were found to exert therapeutic potential to such diseases via ROS depression. For example, phloroglucinol (1,3,5-trihydroxybenzene; 1,3,5-benzenetriol), a bioactive compound, was shown to have antithrombotic and profibrinolytic effects through directly inhibiting ROS production.^{129,130} Chang *et al.*¹³¹ further demonstrated that the effect of phloroglucinol in attenuating arachidonic acid-induced platelet aggregation was closely

related to a decrease in ROS production. Astaxanthin was also proved to have encouraging therapeutic effects on atherosclerosis, probably by inhibiting oxidative stress, including reducing the ROS production.¹³²

H₂O₂ is reported to be involved in the development of atherosclerotic vascular disease and regulates atherosclerotic plaque stability under pathological conditions.¹³³ Therefore, inhibition of the source of H₂O₂ production, such as NOX, is another promising therapeutic strategy. Several agents, such as apocynin and Pefabloc, have been shown to block oxidase assembly.^{134–136} Noticeably, studies have also suggested that the p47phox subunit is most likely a clinically safer target than other NOX subunits that are required for enzyme activity.¹³⁷ Therefore, the p47phox subunit of NOX will be a primary target for treating such diseases in the future.

Conclusions

The field of thrombopoiesis research is currently growing, and our understanding of the molecular basis of megakaryocytopoiesis has progressed substantially, as MKs were first found to give rise to platelets in 1906. The role of ROS in MK biology has increasingly drawn attention in recent years. Despite great advances in the knowledge that ROS has an important role in platelet production and activation, the precise cell signalling events and mechanisms underlying the regulation of ROS are not completely understood.

Other important issues remain to be addressed, and we hope that researchers will be inspired to determine where the boundary lies between the physiological and pathophysiological effects of ROS on thrombopoiesis and platelet activation. These results will provide new insights into ROS-based platelet production. The mechanism through which ROS affect MK biology and cytokine–receptor signalling, including downstream molecules, must be precisely and thoroughly investigated because the current studies on ROS effects on megakaryocytopoiesis are limited. In addition, the study of ROS will certainly provide new therapeutic targets for the treatment of abnormal platelet counts and activation, which is critical for the development of new drugs to treat platelet-related diseases.

Conflict of Interest

The authors declare no conflict of interest.

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