

FORUM REVIEW ARTICLE

Manganese Superoxide Dismutase Regulates a Redox Cycle Within the Cell Cycle

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Abstract

Significance: Manganese superoxide dismutase (MnSOD) is a nuclear-encoded and mitochondria-matrix-localized oxidation-reduction (redox) enzyme that regulates cellular redox homeostasis. Cellular redox processes are known to regulate proliferative and quiescent growth states. Therefore, MnSOD and mitochondria-generated reactive oxygen species (ROS) are believed to be critical regulators of quiescent cells' entry into the cell cycle and exit from the proliferative cycle back to the quiescent state. **Recent Advances/Critical Issues:** Recent evidence suggests that the intracellular redox environment fluctuates during the cell cycle, shifting toward a more oxidized status during mitosis. MnSOD activity is higher in G₀/G₁ cells compared with S, G₂ and M phases. After cell division, MnSOD activity increases in the G₁ phase of the daughter generation. The periodic fluctuation in MnSOD activity during the cell cycle inversely correlates with cellular superoxide levels as well as glucose and oxygen consumption. Based on an inverse correlation between MnSOD activity and glucose consumption during the cell cycle, it is proposed that MnSOD is a central molecular player for the "Warburg effect." **Future Directions:** In general, loss of MnSOD activity results in aberrant proliferation. A better understanding of the MnSOD and mitochondrial ROS-dependent cell cycle processes may lead to novel approaches to overcome aberrant proliferation. Since ROS have both deleterious (pathological) and beneficial (physiological) effects, it is proposed that "eustress" should be used when discussing ROS processes that regulate normal physiological functions, while "oxidative stress" should be used to discuss the deleterious effects of ROS. *Antioxid. Redox Signal.* 20, 1618–1627.

Introduction

THE FIRST EVIDENCE that intracellular oxidation-reduction (redox) reactions regulate cell division dates back to 1931 when Louis Rapkine demonstrated the cyclic pattern of intracellular soluble thiols during mitosis of sea urchin eggs (67). Subsequently, Kawamura and Dan (34) showed that the staining of protein thiols increased in the prophase of sea urchin eggs as the mitotic spindle was assembling. This increase in protein thiols remained high as cells moved into metaphase, followed by a gradual decrease in anaphase, and was almost undetectable in telophase. In synchronous populations of human adenocarcinoma (HeLa) cells, Mauro *et al.* (45) observed that the concentration of non-protein thiols increased approximately 3-fold during mitosis compared with interphase. Using an oxidation-sensitive chemical and flow

cytometry measurements of cell cycle positions, we have demonstrated that the intracellular redox state shifts toward a more oxidized environment during mitosis compared with interphase in synchronized HeLa cell cultures (24). Furthermore, we observed a gradual increase in cellular glutathione (GSH) levels as the cells progressed through the cell cycle (Goswami and Spitz, unpublished observations), which was also consistent with a recent report from Conour *et al.* (13) demonstrating a significant increase in cellular GSH levels during S and G₂ phases of the cell cycle compared with G₁ phase. Moreover, we have shown a transient increase in pro-oxidant levels during the G₁ phase that is required for the mouse embryonic fibroblasts (MEFs) to initiate DNA synthesis. Inhibiting this pro-oxidant event using an antioxidant (N-acetyl-L-cysteine [NAC]) significantly inhibited G₁ cells' entry into S phase (49). Results from these previous studies

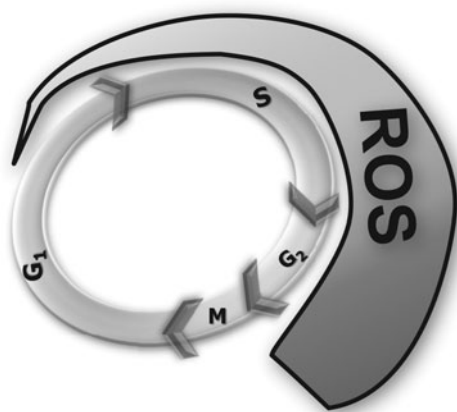


FIG. 1. An illustration showing an increase in intracellular ROS levels during progression from G₁ to S to G₂ and M phases. ROS, reactive oxygen species.

have established the concept of a redox cycle (Fig. 1) within the mammalian cell cycle, coordinating cell cycle progression with cellular metabolism (47).

Cell Cycle Regulatory Proteins

The sequential progression through the cell cycle is regulated by the periodic activation of cell cycle regulatory proteins (Fig. 2). The independent discoveries of cyclins in sea

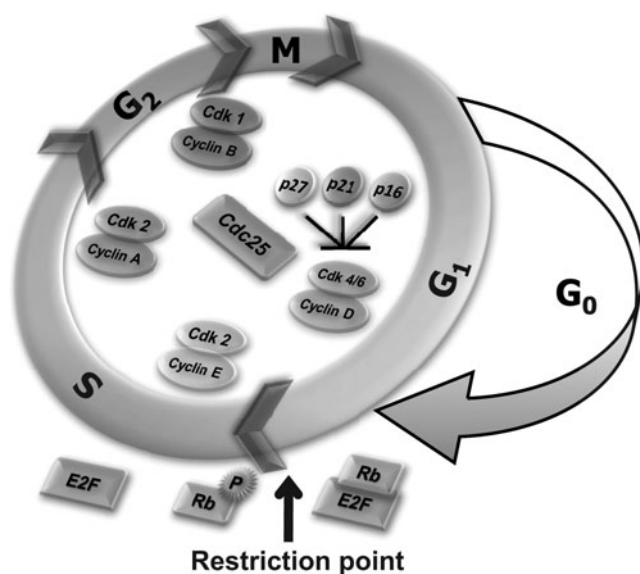


FIG. 2. Cell cycle machinery regulating progression from G₁ to S to G₂ and M phases. Cyclins and CDKs are the positive regulators of the cell cycle. CKIs (p16, p21, and p27) are the negative regulators of the cell cycle. Hypophosphorylated Rb sequesters E2F, which, in turn, restricts entry into S phase. Cyclin D/CDK 4,6 phosphorylates Rb, and this phosphorylation of Rb releases E2F. E2F is a transcription factor that is required for the transcription of multiple S-phase specific genes. Restriction point refers to duration in the G₁ phase, beyond which cells will continue to S phase and cell division independent of the external environment. CDK, cyclin-dependent kinases; CKIs, cyclin-dependent kinase inhibitors; Rb, retinoblastoma.

urchin oocytes, maturation-promoting factors in frog oocytes, and cell-division-cycle proteins in *Saccharomyces cerevisiae* corroborate to the present concept of cyclin and cyclin-dependent kinase (CDK) complexes regulating the cell cycle (18, 29, 44). The cyclin D family (D1, D2, and D3) in combination with CDK4 and CDK6 facilitates the cells' entry from the quiescent (G₀) phase to the G₁ phase of the proliferative cycle (80, 81). Cyclin D1 is the second most commonly amplified gene in the human cancer genome (3). Phosphorylation at Thr286 by glycogen synthase kinase (GSK-3 β) activates proteasomal degradation of cyclin D1 (16). Phosphorylation of GSK-3 β by protein kinase B (AKT) inactivates GSK-3 β kinase activity, thereby stabilizing cyclin D1, which, in turn, facilitates the cells' entry from G₀ to G₁ phase (10). GSK-3 β -independent and mirk/dyrk kinase-dependent phosphorylation at the Thr288 residue can also degrade cyclin D1 (92). Cyclin D1-CDK4/CDK6 phosphorylates the retinoblastoma (Rb) family of proteins (p110, p107, and p130), inactivating Rb and releasing E2F, a transcription factor that activates the transcription of several S-phase specific genes which are required for DNA synthesis (28, 52, 63). The transition from hypo- to hyper-phosphorylation of Rb with the subsequent release of E2F occurs at the "restriction point" (Fig. 2). Arthur B. Pardee defined the "restriction point" as the cells' duration in G₁ phase after which the cells are committed to enter the S phase independent of the external conditions (65). Several recent studies indicate that cyclin D1 has a regulatory role in DNA repair as well as mitochondrial functions that are independent of its CDK4/CDK6-dependent cell cycle regulation (32, 71, 87).

Cyclin E is the next cell cycle regulatory protein that is activated late in the G₁ phase. Cyclin E in association with CDK2 further phosphorylates Rb and inactivates its function (70). Cyclin E is degraded once cells enter S phase. Recent evidence suggests that cyclin E integrates the mitochondrial fusion process to an increase in ATP production and the initiation of DNA synthesis at the G₁/S border (21, 51). The cyclin A/CDK2 kinase complex is activated during S phase, while transit through G₂ phase of the cell cycle is associated with the activation of the cyclin A/CDK1 and cyclin B1/CDK1 kinase complexes. Progression through M phase requires the kinase activity of cyclin B1/CDK1 (66). It is interesting to note that cyclin B1/CDK1-dependent phosphorylation of dynamin-related protein-1 (Ser-585) regulates mitochondrial fission during mitosis (82). These recent reports further suggest the existence of a cross-talk between mitochondrial function and cell cycle regulatory machinery.

The complicated regulation of the cell cycle machinery is further evident from the observations that the kinase activity of cyclin/CDK complexes is regulated by a family of dual specific phosphatases. CDC25 (A, B, and C) dephosphorylates pThr14 and pTyr15 on CDKs, which leads to the activation of cyclin/CDK activity (79). While cyclin/CDKs are the positive regulators of the cell cycle, CDK inhibitors (CKIs) are the negative regulators of the cell cycle (25). CKIs regulate the assembly and activity of the cyclin/CDK complexes. They are broadly classified into two families: INK4 (inhibitors of CDK4) and CIP/KIP (CDK inhibitory protein)/(Kinase inhibitory protein). The INK4-family of proteins (p16 [INK4A], p15 [INK4B], p18 [INK4C], and p19 [INK4D]) bind CDK4/CDK6 and inhibit their kinase activities. The KIP family (p27 and p57) inhibits mainly cyclin E/CDK2 kinase complexes.

The inhibitory effect of p21 has a broader specificity, and it can inhibit all cyclin/CDK activities. Thus, redox control of this complex cell cycle molecular network can influence progression from G₀/G₁ to S to G₂ and M phases.

Cellular Antioxidant Network

The intracellular redox environment is influenced by the production of reactive oxygen species (ROS: superoxide and hydrogen peroxide [H₂O₂]) produced during metabolism and the antioxidant network that removes ROS (Fig. 3). The production of superoxide (O₂^{•-}) and H₂O₂ are the consequence of the incomplete reduction of molecular oxygen (27). Intracellular production of ROS results primarily from the mitochondrial electron transport chain as well as from oxygen-metabolizing flavin-containing proteins, for example, xanthine oxidases, cytochrome P450, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and myeloperoxidase (5, 27, 37, 43, 69, 78, 91).

The cellular antioxidant network includes both the antioxidant enzyme systems and small-molecular-weight antioxidants. Small-molecular-weight antioxidants include GSH, cysteine, ascorbic acid, and α -tocopherol. The antioxidant enzyme network includes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutaredoxin, thioredoxin, and the six-member family of peroxiredoxin (15, 19, 68, 85). SOD converts superoxide to H₂O₂. There are three SOD enzymes in mammalian cells (Fig. 3). MnSOD is a nuclear-encoded and mitochondria-matrix-localized homotetrameric enzyme that converts mitochondrially generated superoxide to H₂O₂ (46). Copper zinc SOD (CuZnSOD) is localized in the cytoplasm, nucleus, and mitochondria intermembrane space (20). The third member of the SOD family, extracellular SOD (EcSOD) is present in the plasma membrane (23). Catalase and GPx 1–4 neutralize H₂O₂ to water. Catalase is localized primarily in the peroxisomes, and different isozymes of GPx are found in most sub-cellular compartments (59, 90). Therefore, changes in the antioxidant network can significantly impact cellular ROS levels, which

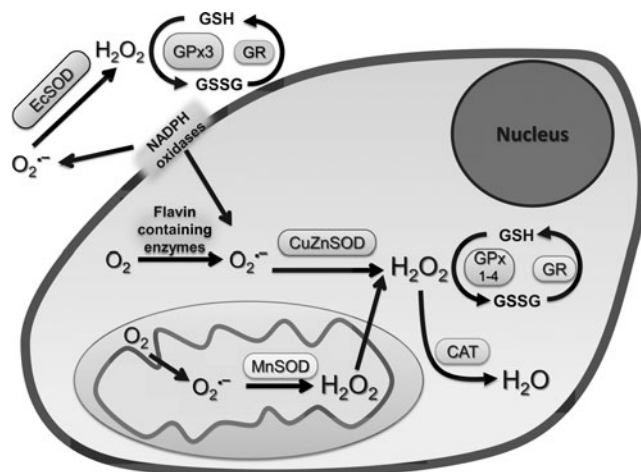


FIG. 3. An illustration showing the intracellular prooxidant and antioxidant network. SOD, superoxide dismutase; Ec, extracellular; GPx, glutathione peroxidase; CAT, catalase; GSH, glutathione; GSSG, glutathione disulfide; GR, glutathione reductase.

can then influence various biological endpoints such as cell proliferation, differentiation, and cell death (75).

Earlier research presented ROS as toxic byproducts of living in an aerobic environment. ROS damage cellular macromolecules (nucleic acids, proteins, and lipids), leading to the activation of the cell death processes (apoptosis, necrosis, mitotic catastrophe, *etc.*). However, both our research and that of others has shown that ROS have normal physiological functions, where they can act as signaling molecules (second messengers) regulating numerous cellular processes, including cellular proliferation (2, 9, 47–49). The mechanisms regulating receptor-ligand and ROS-signaling are distinct. Receptor-ligand signaling involves non-covalent bond formation and complementarity in molecular shape. In contrast, ROS signaling involves covalent bond formation. The second-messenger properties of ROS are believed to be carried out by the thiol-disulfide exchange reactions at specific cysteine residues that are present in various signaling proteins, for example, tyrosine kinases, tyrosine phosphatases, mitogen-activated protein (MAP) kinases, or ion channels (64). The dual function of ROS (signaling molecules and toxins) could result from the differences in their concentrations (threshold), pulse duration (flux), and sub-cellular localization. This hypothesis is consistent with an earlier report by Laurent *et al.* (39), where both the mitogenic and toxic effects of H₂O₂ were clearly demonstrated in NIH 3T3 mouse fibroblasts cultured *in vitro*. H₂O₂ (0.02–0.13 μ M) enhanced proliferation, while treatment with 0.25–2 μ M H₂O₂ resulted in cell death. Therefore, while higher levels of ROS can be toxic, low levels of ROS may serve as signaling molecules regulating numerous cellular processes, including proliferation. Since SODs convert superoxide to H₂O₂, it is reasonable to postulate that SOD can regulate a redox cycle within the cell cycle, thereby influencing redox processes that facilitate progression from G₀/G₁ to S to G₂ and M phases.

MnSOD and the Cell Cycle

MnSOD and cell cycle progression

MnSOD (*sod2*; EC1.15.1.1) is a nuclear-encoded and mitochondria-matrix localized antioxidant enzyme that converts mitochondria-generated superoxide to H₂O₂ (35). Human MnSOD is present in chromosome 6, and it has five exons and two transcripts (1.5 and 4.2 kb) that have the same open reading frame but which differ in the length of their 3'-untranslated region. MnSOD is a homotetramer with a subunit molecular mass of 22 kDa and one Mn³⁺ per monomer. A five-coordinate bipyramidal geometry in each subunit is provided by His-26 and His-74 from the helical domain and Asp-159 and His-163 from the β -sheet domain along with a water molecule (6). Since mitochondria are the major source of intracellular ROS generation, MnSOD is believed to be an important regulator of numerous cellular processes that include cell cycle progression.

In a series of elegant papers, Oberley *et al.* first hypothesized the involvement of MnSOD and ROS during proliferation of normal and cancer cells, cell differentiation, and aging (55, 57, 58). According to the "unified theory" proposed by Oberley *et al.*, a cell division signal is initiated in the plasma membrane, leading to the production of H₂O₂. The membrane-originated signal alters the ratio of cyclic nucleotide levels, and it inactivates catalase and peroxidase, resulting in

the accumulation of H_2O_2 . Signal-initiated accumulation of H_2O_2 stimulates glucose uptake, which then facilitates cellular proliferation. The authors proposed that increased levels of oxygen will halt cellular proliferation, while lower fluxes of superoxide will support proliferation. Furthermore, they proposed that a loss (or decreased levels) of MnSOD combined with increases in superoxide levels will inhibit differentiation and support proliferation (58).

The authors went on to discuss that such a hypothesis would also account for increased glycolysis in cancer cells. Warburg (88) proposed that the increased glycolysis ("Warburg effect") in cancer cells is caused by damage to the respiratory system (*i.e.*, mitochondria). Oberley *et al.* (58) proposed that the damage in cancer cell mitochondria could be caused due to a loss (or decrease) in MnSOD expression. It is anticipated that a decrease in MnSOD expression will increase superoxide (ROS) production, which will lead to an increase in glycolysis and cancer cell proliferation. Although both theories ("Warburg effect" and "unified theory") of cancer cell proliferation have their own merits and limitations, these theories stimulated research efforts to examine a causal relationship between mitochondrial function and cellular proliferation.

MnSOD protein levels and activity were found to be higher in quiescent (G_0) compared with proliferating NIH 3T3 mouse fibroblasts and WI38 human lung fibroblasts (40, 56, 60). NIH 3T3 fibroblasts synchronized to quiescence by serum starvation showed a significant increase in MnSOD expression, which decreased substantially during S phase after the synchronized cells had been allowed to enter the proliferative cycle by adding serum to the culture medium (40). We have shown that MnSOD activity in MCF-10A human mammary epithelial cells decreased from 120 U/mg in quiescent state to 30 U/mg in proliferating cells (74). In synchronized cell populations of MB231 human mammary epithelial cancer cells, MnSOD activity showed a cyclic pattern: G_1 /S phase, 40 U/mg; G_2 +M phases, 26 U/mg; and G_1 phase of the daughter generation, 63 U/mg (74). It is interesting to note that MnSOD activity in the G_1 phase of the daughter generation is comparable to MnSOD activity in the G_1 phase of the parental generation, indicating that the cyclic pattern of MnSOD activity during the parental generation is faithfully preserved in the daughter generation. Overexpression of MnSOD delays cell cycle progression in NIH 3T3 fibroblasts by extending the transit time of G_1 and S phases without any change in G_2 transit time (36).

A regulatory role of MnSOD during the cell cycle is also evident from our studies using both genetic and pharmacological manipulations of MnSOD expression (72, 73, 76, 77). MEFs with wild-type MnSOD showed a typical growth curve consisting of a lag, exponential, and plateau phase (77). Exit from the exponential to plateau phase was delayed in MnSOD heterozygous MEFs, while MnSOD homozygous knockout MEFs failed to exit the proliferative cycle. The inhibition in cellular proliferation of MnSOD heterozygous MEFs was associated with a delayed transit through G_2 +M phases. Overexpression of MnSOD facilitated exit of heterozygous MEFs from the proliferative to the quiescent state. Furthermore, results from our recent study also showed an inverse correlation between MnSOD activity and the percentage of S-phase cells (12).

A lower MnSOD activity was also observed in highly proliferating cancer cells (14, 30, 54, 61, 86, 89). As anticipated,

overexpression of MnSOD suppressed cancer cell proliferation in both cells cultured *in vitro* and mouse xenograft of human cancers (14, 30, 54, 61, 86, 89). MnSOD overexpression-induced suppression of proliferation in androgen-independent human prostate cancer (PC-3) cells was associated with a delay in progression from G_1 to S phase (86). Thus, a lower MnSOD activity is associated with cellular proliferation, whereas a higher MnSOD activity supports cellular quiescence. Furthermore, a cyclic pattern of MnSOD activity during the cell cycle of the parental generation is faithfully replicated in the daughter generation.

The periodic change in MnSOD activity during the cell cycle regulating cell cycle phase-specific response is also evident under conditions of oxidative stress. Ionizing radiation (IR)-induced chromosomal aberration in human lymphocytes was higher in G_2 and M phases, correlating with a decrease in MnSOD activity; while G_0 cells with a higher MnSOD activity correlated with a lower incidence of chromosomal aberration (53). Furthermore, IR-induced chromosomal aberration in G_2 and M phases was suppressed in SOD-treated human lymphocytes (11). High and low LET radiation-induced cell cycle phase-specific toxicity also correlated with the periodic changes in MnSOD activity during the cell cycle. A higher MnSOD activity during G_1 phase was associated with radioresistance, while radiosensitization of G_2 cells correlated with a lower MnSOD activity (4). Consistent with these results, we reported radioresistance of MnSOD overexpressing SCC25 human oral squamous carcinoma cells and MIA PaCa-2 human pancreatic cancer cells (22, 33). MnSOD overexpressing irradiated cells showed a higher percentage of G_2 cells, which was associated with a decrease in phosphorylated ATM and cyclin B1 protein levels. Furthermore, IR-induced G_2 -checkpoint activation was absent in MnSOD homozygous knockout MEFs (17, 26, 41, 62, 83). Inhibition of MnSOD expression in MCF7 human breast cancer cells adapted to low-dose IR decreased cyclin B1, cyclin D1, and p21 expression (26, 41, 62). These results suggest that the cell cycle phase-specific radiation response is regulated *via* MnSOD activity-dependent control of cell cycle gene expression.

Regulation of MnSOD activity during the cell cycle

The mechanisms regulating the cyclic pattern of MnSOD activity during the cell cycle appear to be highly complex (Fig. 4). Forkhead transcription factors (FOXO1 and FOXO3a) have been shown to increase MnSOD transcription during quiescence (1, 38). $NF\kappa B$, a well-studied transcription factor, positively regulates MnSOD transcription. During entry into the proliferative cycle, E2F (transcription factor required for transcription of multiple genes before the initiation of DNA synthesis) competes with the p65 subunit of $NF\kappa B$ and inhibits the binding of p65 to its heterodimer subunit (p50), leading to an inactivation of $NF\kappa B$ transcriptional activity. E2F-induced inactivation of $NF\kappa B$ suppressed MnSOD transcription, which correlated with a lower MnSOD activity during transition from quiescence to the proliferating cycle (84).

We recently demonstrated a preferential abundance of MnSOD transcripts during quiescent and proliferative growth states (12). Human MnSOD has two transcripts (1.5 and 4.2 kb) with the same open reading frame but differing in the length of their 3'-untranslated regions. MnSOD 1.5 kb

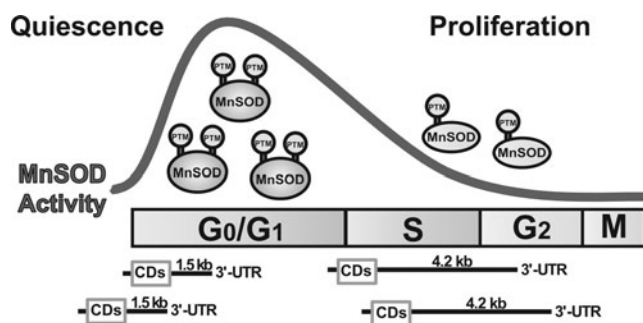


FIG. 4. Regulation of MnSOD expression during quiescence and proliferation. Human MnSOD has two transcripts that carry the same CD but which differ in their 3'-untranslated region. The 1.5 kb transcript is more abundant during quiescence, while the 4.2 kb transcript is enriched during proliferation. MnSOD activity is also regulated by methylation-demethylation PTM. Ser-89 methylation is associated with a higher MnSOD activity during quiescence. CD, coding region; PTM, post-translational modifications.

transcript is more abundant in quiescent cells, which correlated with a higher MnSOD activity (Fig. 4). A higher abundance of the 4.2 kb MnSOD transcript in proliferating cells was associated with a lower level of MnSOD activity (Fig. 4).

The complexity of MnSOD expression during the cell cycle is affirmed from our recent finding of a post-translational modification of MnSOD methylation pattern during quiescence and proliferation (74). Results from tandem mass spectrometry (MS^2) analysis showed that MnSOD is methylated at both lysine (68, 89, 122, and 202) and arginine (197 and 216) residues. Lysine-89 is mono-methylated during quiescence and un-methylated in proliferating cells. Additional results from site-directed mutagenesis demonstrate that lysine-89 methylation significantly enhanced MnSOD activity. These results are supported by computational-based molecular modeling simulations. It is anticipated that lysine and arginine methylation of MnSOD during quiescence will increase the accessibility of the active site and the surface area of positive electrostatic potential around and within the active site. These post-translational modifications could then increase the accessibility of superoxide (negatively charged substrate) to the enzyme active site more during quiescence compared with the proliferative state. This is consistent with our observation of a higher MnSOD activity during quiescence and a decrease in MnSOD activity in proliferating cells (74).

MnSOD Regulates a ROS Switch During the Cell Cycle

MnSOD and ROS (superoxide and H_2O_2) levels during the cell cycle

The cyclic pattern of MnSOD activity during the cell cycle correlates with changes in cellular ROS levels. Flow cytometry measurements of dihydroethidium (DHE) oxidation showed increases in probe-oxidation that is consistent with a significant increase in the steady-state levels of superoxide during proliferation compared with quiescent normal human fibroblasts (74, 77). MitoSOX Red oxidation showed an approximately 4-fold increase in proliferating cells, suggesting that the proliferation-associated increase in superoxide could be of

mitochondrial origin. Indeed, a direct correlation was observed between DHE oxidation and the percentage of S-phase cells. The rate of extracellular H_2O_2 production during proliferation and quiescence showed an opposite effect: 3 fmole $cell^{-1} h^{-1}$ in proliferating cells and 8 fmole $cell^{-1} h^{-1}$ in quiescent cells (77). Accumulation of H_2O_2 exhibited an inverse correlation with the percentage of S-phase cells. These results, combined with the cyclical pattern of MnSOD activity during the cell cycle, suggest that MnSOD activity regulates a mitochondrial "ROS Switch" favoring superoxide-signaling (one-electron reduction reactions) regulating proliferation and H_2O_2 -signaling (two-electron reduction reactions) supporting quiescence (Fig. 5).

MnSOD and cell cycle regulatory proteins

Although the details of the one- and two-electron signaling pathways regulating cell cycle progression (Fig. 5) are not clearly delineated, manipulation of the cellular redox environment is known to affect cell cycle regulatory protein levels. Metal cofactors in cell cycle regulatory kinases and phosphatases could be the site for one-electron reduction reactions, while thiol-disulfide exchange reactions in specific cysteine residues in proteins could be the site for two-electron reduction reactions (7, 8). A decrease in MnSOD activity along with an increase in superoxide levels during proliferation correlates with an increase in cyclin D1 and cyclin B1 protein levels (77). Cyclin D1 and cyclin B1 protein levels decreased when MnSOD activity and H_2O_2 levels increased during quiescence. The failure to exit from the cell cycle in MnSOD heterozygous MEFs was associated with a persistent increase in cyclin D1 and cyclin B1 protein levels (77). An inverse correlation between MnSOD and cyclin D1 was also observed in NAC-treated mouse fibroblasts (47–49).

Overexpression of MnSOD suppressed age-associated accumulation of ROS and p16 as well as stabilized p21 protein levels in quiescent human normal fibroblasts (72, 73, 77). Quiescent fibroblasts kept in culture for a long duration lost their capacity to re-enter the proliferative cycle. This loss in the proliferative capacity of quiescent fibroblasts was associated with increased accumulation of ROS and the CKI, p16. However, overexpression of MnSOD inhibits age-associated

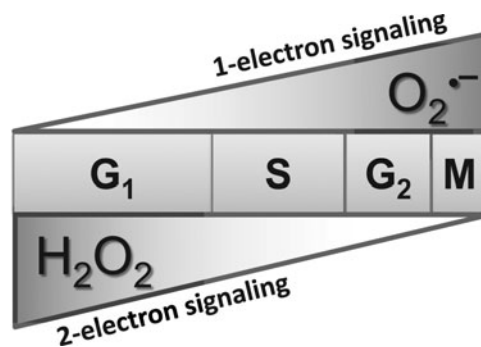


FIG. 5. Superoxide (one-electron) and H_2O_2 (two-electron) signaling during the cell cycle. MnSOD activity is inversely correlated with ROS levels during the cell cycle: A lower level of MnSOD activity is associated with an increase in the steady-state levels of superoxide during S phase, while a higher level of MnSOD activity is correlated with a higher level of H_2O_2 during quiescence. H_2O_2 , hydrogen peroxide.

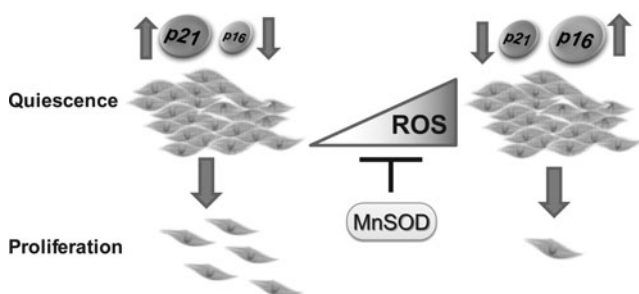


FIG. 6. MnSOD protects cellular proliferative potential. Overexpression of MnSOD suppresses age-associated increase in intracellular ROS levels and p16 accumulation, which was associated with an extension of the proliferative capacity of quiescent fibroblasts.

accumulation of ROS and p16, and it extends the proliferative capacity of quiescent fibroblasts (Fig. 6). Genetic and pharmacological-mediated overexpression of MnSOD has also been shown to increase p27 CK inhibitor protein levels (1, 48). These results led us to the hypothesis that MnSOD activity regulates cell cycle regulatory proteins: cyclins during the proliferative cycle and CKIs during quiescence.

MnSOD is a central molecular player for the Warburg effect

Our recent observations (74, 77) of a cyclical pattern of MnSOD activity during the cell cycle combined with the concept of an “ROS Switch” regulating the cell cycle and an increase in glucose consumption during the cell cycle provide substantial experimental evidence in support of the hypothesis of a “unified theory” of cellular proliferation as proposed by Oberley *et al.* in 1981 (58). Glucose consumption rate (GCR) increased in proliferating cells to support the high demand of bioenergetics and biosynthetic processes of a growing cell population. A direct correlation between GCR and percent S phase was observed in MnSOD wild-type MEFs (74). The GCR of MEFs with 10% S phase was approximately $40 \text{ pg cell}^{-1} \text{ h}^{-1}$, which increased to $120 \text{ pg cell}^{-1} \text{ h}^{-1}$ in cultures with 25% S-phase cells. GCR decreased as MEFs exit the proliferative cycle and entered quiescence. It is worth noting that a correlation between the percentage of S-phase cells and GCR was absent in MnSOD homozygous knockout MEFs. A lack of correlation was also associated with these cells’ inability to exit the proliferative cycle. The inability to exit the proliferative cycle was associated with a relatively constant GCR. Furthermore, we have shown that an increase in MnSOD activity and subsequently a decrease in GCR were accompanied with a decrease in proliferation (74). These results further support our hypothesis that MnSOD regulates a “metabolic switch,” coordinating GCR with cell cycle progression (Fig. 7). We propose MnSOD as a central molecular player that contributes to both the “Warburg effect” and “unified theory” of cellular proliferation.

CuZnSOD and the Cell Cycle

Although the majority of research reports MnSOD-dependent regulation of the cell cycle, adenovirus-mediated overexpression of CuZnSOD suppresses low-density lipoprotein-induced stimulation of human aortic smooth muscle cell

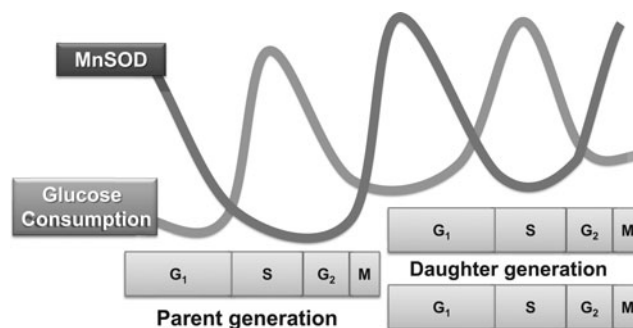


FIG. 7. MnSOD activity regulates a “metabolic cycle” during the cell cycle. An illustration showing the cyclic pattern of an inverse correlation between MnSOD activity and glucose consumption during the cell cycle.

proliferation (42). This suppression in proliferation was associated with an accumulation of cells in G_0/G_1 phase, which correlated with a decrease in cyclins (D1 and E) and CDKs (CDK4 and CDK2), and an increase in CKIs (p21 and p27) protein levels. Fibroblasts incubated with antibodies to CuZnSOD exhibited accelerated proliferation (50). Likewise, fibroblasts isolated from CuZnSOD knockout mice exhibited an approximately 25% slower rate of proliferation compared with wild-type fibroblasts (31). These results suggest that CuZnSOD activity may regulate cell cycle progression *via* ROS signaling of non-mitochondrial origin.

Summary

The mechanisms regulating the cell cycle are complex. A sequential activation of phase-specific cyclin/CDK activity facilitates progression from G_0/G_1 to S to G_2 and M phases. Although the transcriptional, post-transcriptional,

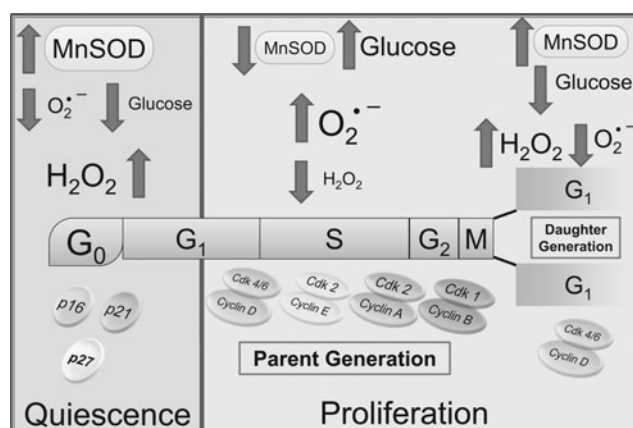


FIG. 8. MnSOD activity regulates a redox cycle within the cell cycle integrating cellular metabolism to the cell cycle regulatory machinery. An illustration representing changes (upward arrow represents increase, while downward arrow represents decrease) in MnSOD activity, glucose consumption, ROS (superoxide and H_2O_2), and cell cycle regulatory proteins during quiescence and progression through the cell cycle of the parental and daughter generations. p16, p21, and p27 are CKIs. Cyclin/CDKs are the positive regulators of the cell cycle, and CKIs are negative regulators. Redox, oxidation-reduction.

translational, and post-translational regulation of cyclin/CDK complexes is well known, interesting questions remain about the cyclic nature of their expression during the cell cycle. The literature discussed here supports the hypothesis that a periodic change in the cellular redox status (a redox cycle) integrates cellular metabolism to the cell cycle machinery during progression from G₀ to G₁ to S to G₂ and M phases.

Since MnSOD is a redox enzyme that regulates mitochondria-generated ROS, changes in MnSOD activity can significantly influence cellular redox status during the cell cycle. We have shown that the cellular redox state shifts toward a more oxidizing environment during S, G₂, and M phases (24). Cellular oxidation state increases three- to fourfold in mitotic cells compared with cells in the G₁ phase. Interestingly, after cell division, the cellular redox state is reset to the redox state of cells in the G₁ phase. Inhibition of this oxidation state before S phase negatively impacts DNA synthesis (49).

Recent evidence (74, 77) suggests that a periodic change in MnSOD activity during the cell cycle may regulate the redox cycle within the cell cycle (Fig. 8). The changes in MnSOD activity during the cell cycle could result from a preferential selection of its two transcripts (12), thereby influencing its translation and protein levels as well as a post-translational modification of methylation-demethylation at specific serine and arginine residues (74). Such an intricate regulation is needed to maintain an appropriate level of MnSOD activity in each cell cycle phase, which, in turn, will maintain a specific threshold of ROS in each phase of the cell cycle, permitting a sequential transition from one cell cycle phase to the next. For example, in order to maintain a higher oxidation state during S, G₂, and M phases, one would expect that MnSOD activity should be lower in these cell cycle phases compared with the G₁ phase. Likewise, it is anticipated that MnSOD activity would be higher in the G₁ phase to facilitate a lower oxidation state. Indeed, this observation led to the notion that MnSOD activity regulates an "ROS switch," facilitating superoxide signaling that promotes proliferation and H₂O₂ signaling which supports quiescence.

The literature discussed here points toward the idea that MnSOD activity regulates a "metabolic switch" during the cell cycle (Fig. 8). It is well known that proliferating cells consume more glucose to meet the high demand of bioenergetics and biosynthetic processes that are required for cell division. As anticipated, GCR increases as cells transit through S to G₂ to M phases, and after mitosis, GCR is reset to cells in the G₁ phase. It is worth noting that this periodic pattern of glucose consumption during the cell cycle is inversely correlated with MnSOD activity (74). Furthermore, genetic manipulations of MnSOD expression inversely affected GCR and cellular proliferation. These exciting observations led us to propose MnSOD as a central molecular player for the "Warburg effect." These observations also provide experimental evidence for the "unified theory" of cellular proliferation put forward by Oberley *et al.* in 1981, linking MnSOD to ROS and glucose consumption, and initiation of cellular proliferation (58).

Future Directions

The concept of a redox cycle integrating cellular metabolism to the cell cycle regulatory machinery is gaining acceptance from the scientific community. We believe that this

concept will also be successful in bridging cell cycle and free radical biology research, which will further advance our understanding of the redox biology of cell cycle regulation. Future research is needed to (i) decipher specific one- and two-electron signaling processes that regulate the cell cycle; (ii) source, pulse duration (flux), and threshold of ROS that regulate specific events during the cell cycle; and (iii) quantitative redox biology to distinguish between ROS levels which are necessary for physiological processes and ROS levels that lead to pathology. Since increasing evidence suggests that ROS have both beneficiary (normal physiological processes) and deleterious (cell death, pathological conditions, *etc.*) effects, we propose that "eustress" should be used when discussing ROS processes which regulate normal physiological functions, while "oxidative stress" should be used to discuss the deleterious effects of ROS.

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Abbreviations Used

CAT = catalase
 CDK = cyclin-dependent kinase
 CKI = cyclin-dependent kinase inhibitors
 CuZnSOD = copper zinc superoxide dismutase
 DHE = dihydroethidium
 EcSOD = extracellular superoxide dismutase
 FoxO = forkhead transcription factor
 GCR = glucose consumption rate
 GPx = glutathione peroxidase
 GSH = glutathione
 GSK = glycogen synthase kinase
 GSSG = glutathione disulfide
 H₂O₂ = hydrogen peroxide
 IR = ionizing radiation
 MEFs = mouse embryonic fibroblasts
 MnSOD = manganese superoxide dismutase
 NAC = N-acetyl-L-cysteine
 NADPH = nicotinamide adenine dinucleotide phosphate
 Rb = retinoblastoma
 Redox = oxidation-reduction
 ROS = reactive oxygen species