Manganese Superoxide Dismutase Regulates a Metabolic Switch during the Mammalian Cell Cycle

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Abstract

Proliferating cells consume more glucose to cope with the bioenergetics and biosynthetic demands of rapidly dividing cells as well as to counter a shift in cellular redox environment. This study investigates the hypothesis that manganese superoxide dismutase (MnSOD) regulates cellular redox flux and glucose consumption during the cell cycle. A direct correlation was observed between glucose consumption and percentage of S-phase cells in MnSOD wild-type fibroblasts, which was absent in MnSOD homozygous knockout fibroblasts. Results from electron paramagnetic resonance spectroscopy and flow cytometric assays showed a significant increase in cellular superoxide levels in S-phase cells, which was associated with an increase in glucose and oxygen consumption, and a decrease in MnSOD activity. Mass spectrometry results showed a complex pattern of MnSOD-methylation at both lysine (68, 89, 122, and 202) and arginine (197 and 216) residues. MnSOD protein carrying a K89A mutation had significantly lower activity compared with wild-type MnSOD. Computational-based simulations indicate that lysine and arginine methylation of MnSOD during quiescence would allow greater accessibility to the enzyme active site as well as increase the positive electrostatic potential around and within the active site. Methylation-dependent changes in the MnSOD conformation and subsequent changes in the electrostatic potential around the active site during quiescence versus proliferation could increase the accessibility of superoxide, a negatively charged substrate. These results support the hypothesis that MnSOD regulates a “metabolic switch” during progression from quiescent through the proliferative cycle. We propose MnSOD as a new molecular player contributing to the Warburg effect. Cancer Res; 72(15); 3807–16. ©2012 AACR.

Introduction

Normal cell proliferation is an orderly transition from quiescence (G0) to proliferation (G1, S, G2, and M phases) and back to quiescence. Such an orderly transition is needed to maintain the regenerative capacity of cells and to avoid aberrant proliferation. Progression from quiescent to proliferative phase is regulated by the sequential activation of cyclin-dependent kinases (CDK; refs. 1, 2). Cyclin/CDKs are the positive regulators of cell-cycle progression, whereas CDK inhibitors are the negative regulators of cellular proliferation. Recent evidence suggests that metabolic activities play a significant role linking cellular metabolism to the cell-cycle regulatory machinery (3–7). Cellular proliferation is accompanied by an increase in the utilization of glucose and glutamine. Aerobic glycolysis coordinates the bioenergetic and biosynthetic demands of rapidly proliferating cells (4, 5). During aerobic glycolysis, glucose is converted to pyruvate, which is then converted to lactate by lactate dehydrogenase. Pyruvate can enter the tricarboxylic acid cycle and mitochondrial electron transport chain leading to the generation of ATP. The third pathway of glucose utilization involves the pentose phosphate pathway, which generates precursor metabolites needed for cellular macromolecular synthesis. Previous studies have established a regulatory role for glucose-6-phosphate dehydrogenase, the rate-limiting step in the pentose phosphate pathway, during proliferation (8, 9). NADPH is one of the products of the pentose phosphate pathway, which is directly coupled to the cellular glutathione and thioredoxin redox systems. It is postulated that increased glycolysis during proliferation could counter a shift in the cellular redox environment facilitating progression through the cell cycle. In fact, we and others have observed a gradual increase in cellular glutathione levels as cells progress through the cell cycle (unpublished observations; ref. 10).

The increase in glutathione during the cell cycle could be in response to a shift in the cellular redox environment toward a more oxidizing environment (11, 12). The redox environment...
of mitotic cells is 3- to 4-fold more oxidizing than G2-cells (11). These previously published results support the hypothesis that a redox cycle within the cell cycle regulates progression from quiescent through the proliferative cycle (13). The cellular redox environment is a balance between the production of reactive oxygen species (ROS: superoxide and hydrogen peroxide) and their removal by the antioxidant network. The mitochondrial electron transport chain is the major generator of cellular ROS. Manganese superoxide dismutase (MnSOD) is a redox enzyme that is encoded by nuclear DNA and localized to the mitochondrial matrix. MnSOD converts mitochondrion-generated superoxide to hydrogen peroxide. Recent evidence suggests that MnSOD activity and mitochondrial generated ROS regulate transitions between the quiescent and proliferative cycle (14-16). MnSOD-dependent regulation of quiescent cells entrance into the proliferative cycle and subsequent exit from the proliferative cycle is associated with changes in levels of cyclins (D1 and B1) and CDK inhibitors (p21 and p16; refs. 14, 16). Cyclin D1 integrates mitochondrial function with DNA synthesis (17). Similarly, mitochondrial fusion is linked to the activation of cyclin E at the G1-S border and entry into S-phase (18). Results presented in this study show: (i) a direct correlation between glucose consumption and percent S-phase in MnSOD wild-type mouse embryonic fibroblasts (MEF); (ii) a periodic change in MnSOD activity during the cell cycle; (iii) an increase in superoxide levels during S-phase; and (iv) a change in MnSOD methylation status in quiescent compared with proliferating cells. These results support the hypothesis that MnSOD regulates a "metabolic switch" during progression from quiescent through the proliferative cycle.

Materials and Methods

Cell culture

Human normal skin fibroblasts (AG01522D, Coriell Cell Repositories), mammary epithelial nontumorigenic (MCF10A) and adenocarcinoma (MB231) cells (American Type Culture Collection, Manassas, VA), wild-type and homozygous knock-out MnSOD MEFs were cultured following our previously published protocols (16, 19, 20). Adenovirus infections and transgene expression were conducted (15, 16, 19, 20), and MnSOD activity was measured using biochemical and gel electrophoresis-based assays (14, 15, 21). Fibroblasts were synchronized by contact inhibition (22). MB231 cells were synchronized at the G1-S border using 2 mmol/L thymidine, washed, and continued in culture for isolation of cells in S- and G2 phases. G1 cells of the daughter generation were obtained by incubating G1-S synchronized MB231 cells with nocodazole (200 ng/mL) and harvesting cells at 4-hour postmitotic shake-off.

Flow cytometric assays

Cell-cycle phase distributions were determined by flow cytometric measurements of DNA content (16). Dihydroethidium (DHE), MitoSOX-Red, and MitoTracker-Green fluorescence were used to probe for cellular and mitochondrial ROS levels and mitochondrial mass (15, 16, 22). Mean fluorescence intensity was calculated using FlowJo software (Tree Star, Inc.). Auto-fluorescence of cells was used for background correction; fold change calculated relative to control or G1 cells.

Immunoprecipitation assay

Immunoprecipitation was done following the manufacturer’s supplied protocol (Direct IP from Pierce). Rabbit polyclonal antibody against methylated-lysine (Abcam Inc.) was coupled to beads (AminoLink Plus Coupling Resin), and incubated with 500 µg of protein extracts. Bound proteins were eluted and MnSOD was identified in the immunoprecipitates via immunoblotting.

Site-directed mutagenesis

QuickChange II Kit (Stratagene) was used to mutate lysine 89 and lysine 202 of MnSOD to alanine. pShooter (Invitrogen) expression vectors carrying human MnSOD cDNAs with wild-type and lysine-to-alanine mutations were transfected into MnSOD+/− MEFs. MnSOD expression was measured using Western blotting and activity assays.

Electron paramagnetic resonance spectroscopy

Cells were incubated with PBS containing 100 mmol/L 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and electron paramagnetic resonance (EPR) spectra recorded using a Bruker EMX spectrometer with a magnetic field modulation frequency of 100 kHz; modulation amplitude, 1.0 G; scan rate, 60 G/21 s (15, 16). Spectra were results of 40 signal-averaged scans collected over about 20 minutes. EPR peak heights (WinEPR software) were normalized to cell number. The specificity of the superoxide origin of the signal was determined by preincubating cells with CuZnSOD (1,000 units/mL).

Glucose and oxygen consumption consumption assays

Cultures were incubated for 4 to 6 hours with Dulbecco's Modified Eagle Medium and glucose concentration was measured using a Bayer Glucometer Elite with Bayer Ascensia Elite Blood glucose test strips (23). Glucose consumption rate (GCR) was calculated per cell, and fold-change was determined relative to the GCR of G1 cells.

Oxygen consumption rate (OCR) of cells was measured polarographically (Yellow Springs Instrument Co.). Measurements were conducted at 37°C on 3 mL samples, air-saturated culture media without serum with 5 to 8 x 10⁶ cells. OCR was expressed as atomoiles of oxygen consumed per cell per second, using an initial oxygen concentration of 192 µmol/L (24).

Mass spectrometry

Total cellular protein extracts prepared from quiescent and proliferating cultures of normal human fibroblasts were resolved using gel electrophoresis. Coomassie Blue stained gel slices were excised and subjected to tandem mass spectrometry (MS²) analysis.

Molecular modeling

Molecular modeling simulations were conducted using Sybyl-X software (Version 1.2; Tripos, Inc.). The crystallographic coordinates of the 2.3 Å human MnSOD structure (PDB: 2GDS), were obtained from the RCSB Protein Data Bank (25), and the Asp54 residues in the tetramer were replaced with His residues. The MMFF94s force-field and MMFF94 charges were

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applied to the unmethylated MnSOD tetramer and the structure was minimized using the Powell method (1,000 iterations; termination gradient of 0.001 kcal/mol; ref. 26). The proliferative and quiescent forms of MnSOD were further minimized using the Powell method to examine structural changes induced by lysine and arginine mono- and dimethylation.

Statistical analysis

Statistical analysis was done using the 1- and 2-way ANOVA with Tukey honestly significant difference test (SPSS computer software version 11.0).

Results

MnSOD activity regulates glucose consumption during cellular proliferation

Cellular proliferation is accompanied by an increase in glucose consumption to support the high demand of bioenergetics and biosynthetic processes. MnSOD activity and mitochondrial ROS have been shown to regulate transitions between the quiescent and proliferative states. To determine if MnSOD activity coordinates GCR during transition from the quiescent to the proliferative state, we initially determined if MnSOD activity coordinates GCR during transition from the quiescent to the proliferative state, we initially determined if GCR varies during the cell cycle. GCR increased approximately 6-fold in S- and G2 cells compared with G1 cells (Fig. 1A). The OCR of G1 cells was determined to be approximately 50 amol/cell/s, and G2 cells exhibited more than 100 amol/cell/s, a 2-fold increase in OCR (Fig. 1B). GCR in MnSOD wild-type MEFs exhibited a direct correlation with the percentage of S-phase cells ($R^2 = 0.8$; Fig. 1C). GCR was approximately 40 pg/cell/h in cultures with 10% S-phase, and 120 pg/cell/h in cultures with 25% S-phase cells (Fig. 1C). GCR decreased as cells exit the proliferative cycle and entered quiescence (Fig. 1D). It is interesting to note that such a correlation between the percentage of S-phase cells and GCR was absent in homozygous knockout MnSOD MEFs ($R^2 = 0.0003$; Fig. 1E). As reported previously, MnSOD$^{-/-}$ MEFs did not exit the proliferative cycle (16), and the inability to exit the proliferative cycle was associated with a relatively constant GCR (Fig. 1F).

To further determine whether MnSOD activity regulates GCR, MnSOD$^{-/-}$ MEFs were infected with 100 MOI (multiplicity of infection) of AdMnSOD (an adenoviral construct containing the cDNA for MnSOD). AdMnSOD infection increased MnSOD protein levels and activity (Fig. 4E, Supplementary Fig. S1). The GCR in control and AdBgl infected cells at 96-hour postinfection was approximately 50 pg/cell/h, and a 2-fold decrease in GCR was observed in AdMnSOD infected cells (Fig. 2A, left). An increase in MnSOD activity and subsequently a decrease in GCR were accompanied with a decrease in cell number (Fig. 2A, right). The generality of this phenomenon was further evaluated by repeating the experiment using MB231 cells. GCR in control and AdBgl infected MB231 cells was approximately 65 pg/cell/h and overexpression of MnSOD suppressed GCR by approximately 2-fold (Fig. 2B, left).

Figure 1. A direct correlation between glucose consumption and percentage of S-phase cells in MnSOD wild-type MEFs. Quiescent MEFs were replated at a lower cell density and harvested at different times for flow cytometric measurements of cell-cycle phases. Cells from replicate dishes were used for glucose (A) and oxygen (B) consumption assays. *, statistical significance relative to G0 cells; $n = 3$, $P < 0.05$. MEFs with wild type (+/+) and knockout (-/-; E and F) MnSOD were cultured to obtain cells with varying percentage in S-phase. Cells from replicate dishes were used to measure glucose consumption rate. Regression plots show the correlation between the rate of glucose consumption and percentage of S-phase. In a separate set of experiments, cell numbers and glucose consumption rates were determined at different days postplating (D and F).
As previously shown (Fig. 2A, right), overexpression of MnSOD and subsequent decrease in GCR inhibited cell growth (Fig. 2B, right). Similarly, a decrease in MnSOD activity in normal human fibroblasts overexpressing a dominant-negative form of MnSOD increased GCR and percentage of S-phase cells (Supplementary Fig. S2). These results support the hypothesis that MnSOD activity regulates a “metabolic switch,” facilitating a redox environment that is conducive for cell-cycle progression.

**Cell-cycle phase–specific accumulation of ROS levels**

MnSOD is a redox enzyme that is known to regulate the cellular redox environment by converting superoxide to hydrogen peroxide. To determine if changes in GCR and MnSOD activity (Fig. 4) during the cell cycle were associated with changes in the cellular redox environment, flow cytometry and EPR measurements of cellular ROS levels were conducted in synchronized MEFs. The percentage of G0–G1 cells at the time of postreplating was approximately 80% and decreased to 20% at 20-hour postreplating when 60% of the cells progressed to S- and G2 phases (Fig. 3A). Cellular ROS levels, measured by DHE oxidation, increased approximately 3-fold at 20- to 24-hour postreplating compared with the cells at the time of replating. A 2- to 3-fold increase in MitoSOX-oxidation, an indicator of an increase in mitochondrial ROS levels (15, 16, 22), was observed in S- and G2 cells compared with G1 cells (Fig. 3B).

It is interesting to note that MitoSOX fluorescence did not show any significant change during the cell cycle of MnSOD cDNA overexpressing versus control and AdBglII-infected cells; n = 3, P < 0.05.

These results suggest that MnSOD activity shifts the cellular (presumably mitochondrial) redox environment toward a more oxidizing environment as cells progress through the cell cycle.

Flow cytometric measurements of DHE oxidation were repeated to distinguish superoxide from other oxidants (27). These results indicate that the increase in DHE oxidation during S- and G2 phases was primarily because of an increase in cellular superoxide levels (Supplementary Fig. S4). These results are also consistent with results obtained from EPR spectroscopy (Fig. 3C and D). A 1:2:2:1 spectrum of DMPO-ÖH was observed in synchronized S-phase cells. EPR peak height measurements showed an approximately 4-fold increase in DMPO-ÖH in S-phase compared with G1 phase cells. A significant suppression of EPR peak height in S-phase cells pretreated with SOD suggests that the observed DMPO-ÖH species is derived from superoxide.

**Periodic changes in MnSOD activity during the cell cycle**

Mitochondria are the major source of cellular ROS and MnSOD activity is known to regulate mitochondrial ROS levels. To determine if MnSOD activity itself varies during the cell cycle, synchronized MEFs representative of G1, S-, and G2 phases were assayed for MnSOD activity (21). MnSOD activity was higher in G1 phase and decreased significantly as cells progress through S- and G2 phases (Fig. 4A). The periodic changes in MnSOD activity were not because of a corresponding change in MnSOD protein levels (Fig. 4B). To determine the generality of this observation, MnSOD activity was measured in synchronized cell populations of MB231 (Fig. 4C). MnSOD activity in the G1-S synchronized cells was approximately 44 U/mg protein, which decreased to 30 and 26 U/mg protein in S- and G2 phases, respectively. Surprisingly, after cell division, MnSOD activity in the G1 phase of the daughter generation increased to 63 U/mg protein. Furthermore, MnSOD activity in quiescent MCF10A cells was approximately 120 U/mg protein, which significantly decreased to 30 U/mg protein at 24-hour postreplating when approximately 20% cells progressed to S-phase (Fig. 4D). These results showed a periodic change in MnSOD activity during the parental generation that is faithfully preserved in the daughter generation.

**Posttranslational modifications of MnSOD during quiescence and proliferation**

Because the periodic changes in MnSOD activity through the cell cycle were not because of change in its protein levels (Fig. 4B), we investigated whether these changes were related to a posttranslational modification of the protein. First, quiescent and proliferating cultures of MnSOD cDNA overexpressing MEFs were infected with adenoviruses containing cytomegalovirus (CMV) promoter–driven mouse or human MnSOD cDNA. Cyclin D1 protein levels were assessed to evaluate cellular quiescence and proliferation. MnSOD protein levels were comparable in AdMnSOD-infected quiescent and proliferating cells (Fig. 4E). However, MnSOD activity showed a significant increase in quiescent compared with proliferating cells (Fig. 4F and G). These results indicate that a posttranslational modification of...
MnSOD regulates its activity during transitions between quiescence and proliferation.

To characterize specific posttranslational modifications of MnSOD total cellular proteins isolated from quiescent and proliferative cultures were resolved using 1-dimensional gel electrophoresis and Coomassie-stained bands corresponding to MnSOD were excised for tandem mass spectrometry (MS2) analysis. The MS2 spectra were evaluated for common posttranslational modifications such as phosphorylation, acetylation, methylation, SUMOylation, and ubiquitination. Methylation was identified as the only posttranslational modification of MnSOD during quiescence and proliferation (Supplementary Fig. S5A). MnSOD is methylated at both lysine (68, 89, 122, and 202) and arginine (197 and 216) residues (Fig. 5A). In quiescent fibroblasts, MnSOD is dimethylated at K68 and K122, and monomethylated at K89 and K202. Arginine is dimethylated at R197 and monomethylated in R216. In proliferating cells, MnSOD is monomethylated at K68, whereas K89 and K202 were unmethylated. MnSOD K122- and R197-dimethylation did not change between quiescent and proliferative growth stage. R216, which was monomethylated in quiescent cells, became dimethylated in proliferating cells. It is interesting to note the conservation of MnSOD lysine (68, 89, 122, and 202) and arginine (R197 and 216) among different species (Supplementary Fig. S5B).

The MS2 results were validated using an immunoprecipitation-immunoblotting assay (Fig. 5B and C). Total cellular protein extracts prepared from quiescent and proliferating fibroblasts were incubated with antibodies against methylated-lysine (Abcam). An immunoblotting assay was conducted to identify MnSOD in the immunoprecipitates. Consistent with the MS2 results, results from the immunoprecipitation-immunoblotting assay identified MnSOD as being methylated in both quiescent and proliferating fibroblasts (Fig. 5B). The amount of the methylated form of MnSOD seems to decrease in proliferating versus quiescent fibroblasts. Comparable results were also observed in MnSOD-overexpressing quiescent and proliferating MnSOD+/− MEFs (Fig. 5C).

The role of methylation regulating MnSOD activity was further evident from the results shown in Fig. 5D. Site-directed mutagenesis was used to mutate lysine (89 and 202, individually as well as double mutation; Supplementary Fig. S6) to alanine. MnSOD protein levels and activity were measured in MnSOD+/− MEFs transfected with pShooter plasmid DNAs containing wild-type and K-to-A mutant carrying human MnSOD cDNAs. It is interesting to note that although MnSOD protein was present in cells transfected with wild-type and K-to-A mutant carrying human MnSOD cDNAs, K89A mutation significantly decreased MnSOD activity (Fig. 5D). The specificity of K89-methylation regulating MnSOD activity was also evident in cells transfected with the K89A-K202A double mutation. Furthermore, the rate of glucose consumption increased in cells expressing K89A-MnSOD (122 pg/cell/h) compared with wt-MnSOD (87 pg/cell/h). These results show that (i) methylation status of K89 has a significant role in regulating MnSOD activity and (ii) MnSOD activity influences glucose consumption.
Results from the molecular biology approach were comparable to computer modeling of MnSOD-methylation influencing its activity. Models of methylated tetrameric MnSOD were constructed using computational-based molecular modeling, based on our mass spectrometry data of the methylated state of MnSOD during proliferation and quiescence (Supplementary Fig. S7A and S7B). The methylated residues (Lys68, 89, 122, and 202; Arg197 and 216) are well distributed in each monomeric unit of the MnSOD tetramer. Although no large global changes in conformation were observed between the proliferative and quiescent MnSOD models, energy minimization of these models revealed conformational differences in and around the active site, which may modulate the accessibility of substrates and enzyme activity. In the quiescent MnSOD-methylation model, the Mn ion in the active site was more accessible than in the proliferative MnSOD-methylation model (Fig. 6A). In addition, changes in electrostatic potential were also observed around the active site of quiescent MnSOD-methylation model (Fig. 6B). The Lys68 and 89 residues within the monomer and the residues Arg197 and Lys202 from the adjacent monomer were in close proximity to the active site, but are not close enough to sterically affect the accessibility of the substrate. However, the differences in the methylation of residues in proliferative and quiescent MnSOD seem to induce distinct conformational changes in the accessible surface and the electrostatic potential around the active site. The overall contraction of the catalytic residues H50, H98, D183, and H187 around the Mn ion in the active site of the quiescent MnSOD model compared with the proliferative model (Supplementary Fig. S7C) seems to enlarge the entrance to active site cavity, which in addition to increasing the accessibility to the Mn ion also increases the surface area of positive electrostatic potential around the Mn ion. Furthermore, differences in the orientation of amino acid side chains, particularly His50 and 54, in the MnSOD models may also contribute to the enlargement of the active site cavity and changes in electrostatic potential. Therefore, these methylation-dependent changes in MnSOD conformation and electrostatic potential during quiescence may increase the accessibility of superoxide, a negatively charged substrate, to the enzyme active site.

Discussion

Recent evidence suggests that a redox cycle within the mammalian cell cycle could coordinate cellular metabolism to the cell-cycle regulatory machinery (13, 28). We have shown previously that MnSOD activity and mitochondrial generated ROS regulate transitions between quiescent and proliferative states favoring superoxide signaling that facilitates proliferation and hydrogen peroxide signaling that supports quiescence (15, 16). Results from this study showed a significant increase in cellular superoxide levels during S-phase, which correlated with a decrease in MnSOD activity and increase in GCR and OCR. The periodic change in MnSOD activity during the cell cycle.
Aerobic glycolysis was originally reported by Warburg as a way of superoxide-signaling needs to be investigated further, we speculate that a shift toward a more oxidizing environment during S- and G2 phases, yet the fold-change shifts more toward glycolysis (6-fold increase) than respiration (2-fold increase). Although the downstream pathway of superoxide-signaling needs to be investigated further, we speculate that a shift toward a more oxidizing environment during S- and G2 phase could facilitate 1- and 2-electron reduction reactions in preparation for a successful cell division. Metal cofactors in cell-cycle regulatory kinases and phosphatases could be the site for 1-electron reductions, whereas cysteine residues in proteins could be the site for 2-electron reductions (13, 16, 28, 32, 33). The 2-electron reduction reactions of CDC25C has been shown to regulate its phosphatase activity (34). In fact, a previous study used bioinformatics to predict that the thiol-disulfide exchange reactions of cell-cycle regulatory proteins could be maximal in G2 cells compared with G1 cells (10). Thus, periodic changes in ROS generated from metabolic processes could coordinate with the redox-sensitive cell-cycle pathways facilitating progression through the cell cycle.

Mitochondria are the major source of cellular ROS generation. Results presented in Figs. 1 and 2 suggest that MnSOD activity could regulate GCR during proliferation. A direct correlation was observed between GCR and the percentage
of S-phase cells in \(\text{MnSOD}^{+/+}\) MEFs (Fig. 1C). Such a correlation was absent in \(\text{MnSOD}^{-/-}\) MEFs (Fig. 1E). Overexpression of MnSOD in \(\text{MnSOD}^{-/-}\) MEFs resulted in a decrease in GCR and cell proliferation (Fig. 2). Results presented in Fig. 1D and F as well as our earlier published results (16) showed that \(\text{MnSOD}^{-/-}\) MEFs failed to exit the proliferative cycle; whereas, \(\text{MnSOD}^{+/+}\) cells exited from the proliferative cycle and entered into quiescence. The rate of GCR decreased as \(\text{MnSOD}^{+/+}\) MEFs entered quiescence. It is interesting to note that the inability of \(\text{MnSOD}^{-/-}\) MEFs to exit the proliferative cycle was associated with a relatively constant GCR. These results further support our hypothesis that MnSOD regulates a metabolic switch during the cell cycle.

Cancer cells, in general, exhibit a significant decrease in MnSOD activity, and overexpression of MnSOD delays cancer cell proliferation (35–39). It is worth noting that overexpression of MnSOD in rapidly proliferating MB231 cells also showed a decrease in GCR and an inhibition in cellular proliferation (Fig. 2B). MnSOD activity in MB231 cells is 17 U/mg and cell population doubling of 23 hours (40). MCF10A is a nonmalignant human mammary epithelial cell line with high MnSOD activity (36 U/mg) and a cell population doubling time of 31 hours (40). The difference in cell population doubling time correlated with 24% S-phase in MCF10A and 43% S-phase in MB231 cells (41). It is interesting to note that the GCR in MCF10A cells was 33 pg/cell/h (23) compared with 65 pg/cell/h in MB231 cells (Fig. 2B). Taken together, these results clearly showed an inverse correlation of MnSOD activity with GCR, and with the percentage of S-phase cells. There is a direct correlation between MnSOD activity and cell doubling time, higher activity was associated with an increase in cell doubling time, whereas lower MnSOD activity decreases cell doubling time. These previously published results combined with the results presented here further support the hypothesis that MnSOD regulates a "metabolic switch" coordinating GCR with cell-cycle progression.

MnSOD activity exhibited a significant change during the cell cycle (Fig. 4). MnSOD activity in S-phase fibroblasts and MB231 cancer cells was significantly lower than cells in G1 phase (Fig. 4A and C). Following cell division, MnSOD activity increased again in G1 phase of the daughter generation. Overexpression of MnSOD in quiescent and proliferating fibroblasts also showed growth-state related variations in MnSOD activity (Fig. 4E and F), indicating a posttranslational modification of MnSOD regulating its activity. Results from mass spectrometry analysis revealed a complex pattern of lysine and arginine methylation of MnSOD in quiescent and proliferating fibroblasts (Fig. 5A, Supplementary Fig. S5). Consistent with the mass spectrometry analysis, methylation of MnSOD is also evident from the results obtained from the immunoprecipitation–immunoblotting assay (Fig. 5B and C). Results from site-directed mutagenesis show that K89A mutation significantly decreased MnSOD activity (Fig. 5D), suggesting that K89-methylation status influences MnSOD activity. Computational-based molecular

![Figure 6](image_url)
modeling simulations based on the mass spectrometry data indicate that lysine and arginine methylation of MnSOD during quiescence would increase the accessibility of the active site and the surface area of positive electrostatic potential around and within the active site (Fig. 6, Supplementary Fig. S7). These changes could increase the accessibility of superoxide, a negatively charged substrate, to the enzyme active site more during quiescence compared with the proliferative state.

In summary, our results support the hypothesis that MnSOD regulates a "metabolic switch" during the cell cycle. Loss of this MnSOD-dependent metabolic control could lead to aberrant proliferation. We propose MnSOD as a new molecular player that contributes to the Warburg effect.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: E.H. Sarsour, P.C. Goswami
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.H. Sarsour, A.L. Kalen, Z. Xiao, T.D. Veenstra, L. Chaudhuri, P. Reigan, G.R. Buettner

References
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