The Anti-Aging Effects of Patented* Hydroxytyrosol

Nancy B. Ray, PhD; Darlene McCord, PhD FAPWCA

Maintaining the regenerative capacity of normal cells is critical for tissue renewal and repair. Human somatic cells have a finite lifespan or replicative senescence that is defined by a finite number of cell divisions prior to irreversible cell cycle and growth arrest. Most eukaryotic cells including human cells spend the majority of their lifespan in a quiescent state. Cellular quiescence is a reversible growth state that is highly essential for cell and tissue renewal.

Quiescent (G_0) or non-dividing human somatic cells are known to possess age-dependent loss of regenerative capacity. The mechanisms that regulate the entry into, maintenance of, and exit from quiescence are poorly understood but increased understanding of these mechanisms should provide insights into the process of aging. [1] The antioxidant, hydroxytyrosol derived from olives (*Olea Europaea*), was used to investigate some of these mechanisms.

The free-radical theory of aging states that organisms age because cells accumulate free radical damage over time. It is thought that there is a shift in the cellular redox environment facilitating progression through the cell cycle. A regulatory role of reactive oxygen species (ROS), a major source of free radicals, is included in this

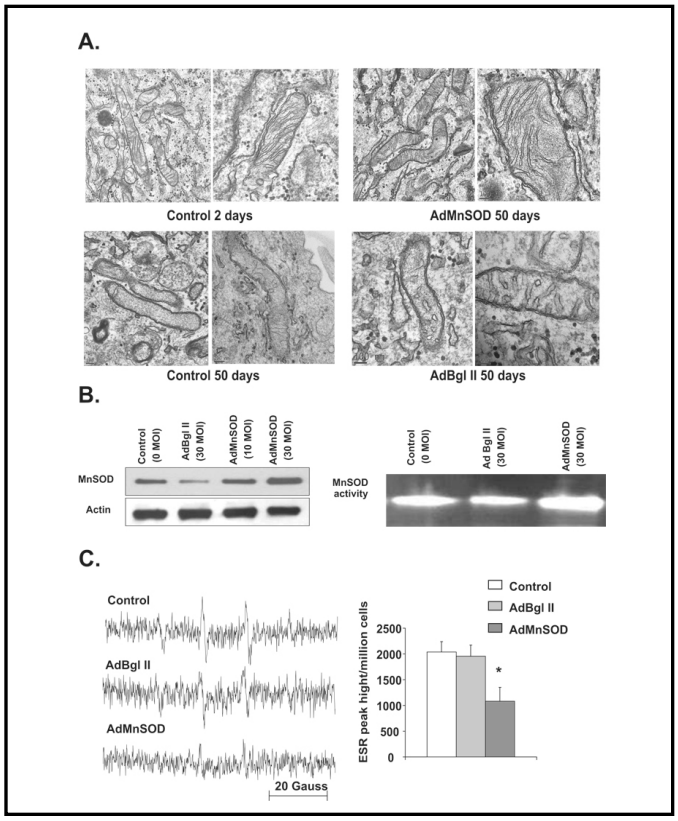

Figure 1: A. Transmission electron microscopy photos of mitochondria in young (2 days) and old (50 days) contact inhibited quiescent NHFs cultured in 21% oxygen environment (left panels). 2 day old quiescent NHFs overexpressing MnSOD (AdMnSOD) and with control vector (AdBglII) were cultured for a total of 50 days. Cells were collected by scraping and processed for visualization of mitochondrial morphology using transmission electron microscopy (right panels); scale ranges from 100 nm to 0.2 µm. Representative of 2 or more experiments. **B**. Total cellular protein extracts were analyzed for MnSOD protein levels by immunoblotting (***Left panel***), and MnSOD activity using native gel-electrophoresis assay (***Right panel***). **C**. Electron resonance spectroscopy measurements of cellular ROS levels in control, AdBgl II, and MnSOD overexpressing (AdMnSOD) quiescent NHFs; ***Left panel***: representative ESR spectra, ***Right panel***: ESR peak height per million cells. Asterisk indicates significant difference between MnSOD overexpressing NHFs compared to control, and cells with vector control (AdBgl II). n=3, $p < 0.05$.

*McCord Research has three U.S. and worldwide patents pending on this research

theory. ROS can be generated exogenously or endogenously, however, mitochondrial metabolism is the main source of endogenous ROS. The free radical theory of aging is also known as the error theory of aging because it describes age-associated accumulation of damage to cellular macromolecules and organelles (such as mitochondria). It was hypothesized that since hydroxytyrosol scavenges free radicals, it may also help protect against aging.

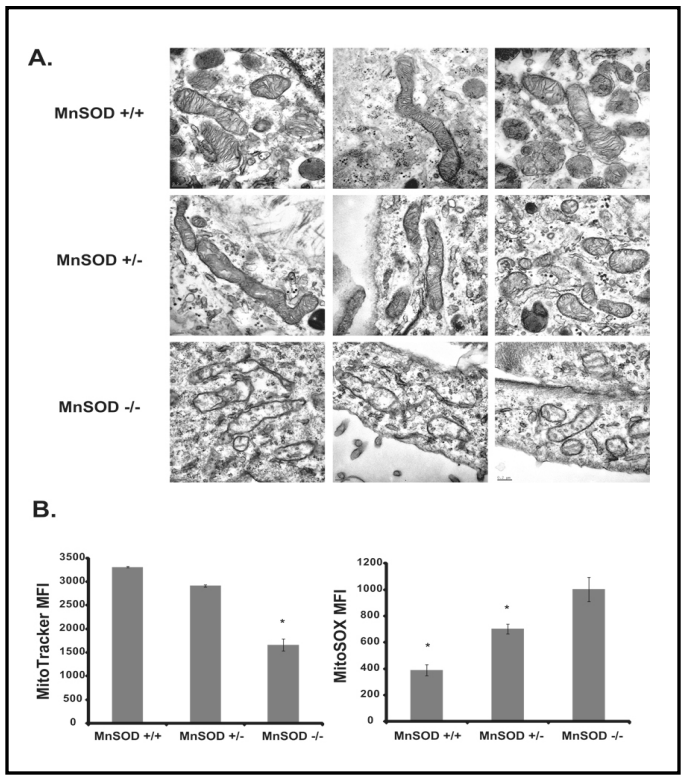

Figure 2: **A**. MnSOD genotype MEFs were cultured in 4% oxygen environment and harvested for transmission electron microscopy visualization of mitochondria morphology; scales range from 100 nm to 0.2 µm. Representative of 2 or more experiments. **B**. MnSOD genotype MEFs cultured in 4% oxygen environment were incubated with MitoTracker (**left panel**), and MitoSox (**right panel**). Fluorescence was measured by flow cytometry. Asterisks (left panel) indicate significant difference in MitoTracker fluorescence in MnSOD (−/−) compared to (+/+) and (+/−) MEFs; asterisks in right panel represent significant difference in MitoSOX fluorescence in MnSOD (+/+) and (+/−) compared to MnSOD (−/−) MEFs; n=3, $p<0.05$.

Eukaryotic cells including human cells have evolved defense mechanisms to protect themselves from ROS-induced oxidative damage, however, there is still a significant amount of ROS accumulation and oxidative damage that occurs during cellular and organism aging. ROS defense mechanisms include cellular antioxidant enzymes, such as superoxide dismutases. One antioxidant enzyme in particular, manganese superoxide dismutase (MnSOD), is critical for the removal of superoxide generated from mitochondria. It has been shown that overexpression (greater than normal) of MnSOD protein protects quiescent normal human fibroblasts (NHF) derived from skin, against age-associated loss in proliferative capacity.[2]

An increase in ROS levels and subsequent changes in mitochondrial morphology of aged quiescent cells could result from an increase in the production of ROS or a decrease in ROS removal due to changes in cellular antioxidant capacity. The hypothesis that MnSOD activity protects mitochondria from age-associated damage and preserves the chronological lifespan of quiescent fibroblasts was investigated by Dr. Ehab Sarsour at the University of Iowa, funded by McCord Research.[3-7] It was demonstrated that overexpression of MnSOD protects mitochondria from age-associated abnormalities. MnSOD overexpressing quiescent cells also exhibited significantly less cellular ROS levels compared to controls.

The effects of differential expression of MnSOD were further investigated in murine embryonic fibroblasts (MEFs) from mice genetically constructed to not express MnSOD (knockout mice). Knockout (gene is "knocked out") mice

that are homozygous are described as MnSOD (-/-), or heterozygous (+/-) if the gene in only one allele is knocked out. Consistent with the results from MnSOD overexpression, knockout and heterozygous mice showed varying levels of mitochondrial structural damage, whereas normal mice (MnSOD+/+) showed none. A mitochondrial-specific dye was used to measure the abundance of mitochondria, which was lower in MEFs with lower expression of MnSOD (MnSOD (+/-) and MnSOD (-/-)), suggesting that MnSOD protects mitochondria from damage and subsequent loss from cells.

A regulatory role of mitochondrial ROS in cellular aging suggested that the oxygen environment could be critical in regulating chronological lifespan (duration of quiescence in

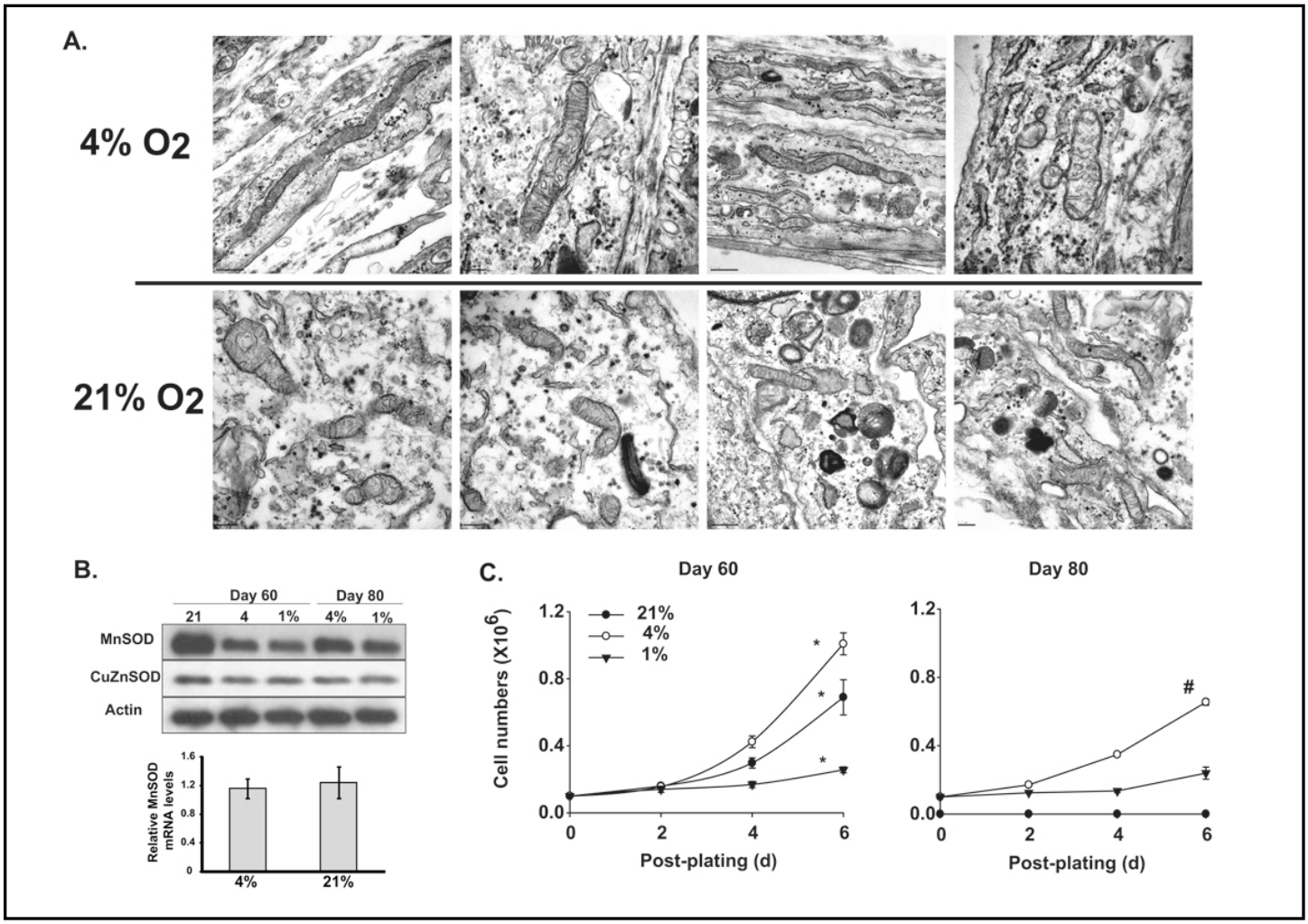

Figure 3: **A.** Transmission electron microscopy visualization of mitochondrial morphology in quiescent NHFs cultured in 1, 4, and 21% oxygen environment. Scales range from 100 nm to 0.2 µm. Representative of 2 or more experiments. **B. Upper panel**: total cellular protein extracts were analyzed for MnSOD and CuZnSOD (for comparison) protein levels by immunoblotting. Actin levels were used for loading correction. **Lower panel**: Relative MnSOD mRNA levels in 12 days quiescent NHFs in 4% vs. 21% oxygen tension using real-time PCR as described in Materials and Methods. Relative MnSOD mRNA levels were calculated relative to 2-day quiescent NHFs. **C**. Growth characteristics of NHFs replated from 60 and 80 days quiescent NHFs cultured in 1, 4, and 21% oxygen environment. Replated cells were cultured in the same oxygen environment and cell numbers counted at indicated times. Asterisks indicate significant difference among different oxygen concentrations; # indicates significant difference in 80 days compared to 60 days quiescence; n=3, $p < 0.05$.

which normal cells retain the capacity to reenter the proliferative cycle). Quiescent NHFs cultured in a 21% oxygen environment were compared to cells cultured in a 4% and 1% oxygen environment (negative control). It was found that lower oxygen (4%) protected quiescent NHFs from age-associated abnormalities in mitochondrial morphology and loss of proliferative capacity. Growing evidence links antioxidants with a decreased risk of several aging-related health concerns.[8] The antioxidant properties of hydroxytyrosol have been widely investigated, however, the exact mechanisms of its hypothesized anti-aging activities are not known. Therefore, the effects of hydroxytyrosol on MnSOD activity and mitochondrial ROS accumulation were examined. Hydroxytyrosol was found to increase the activity of MnSOD.

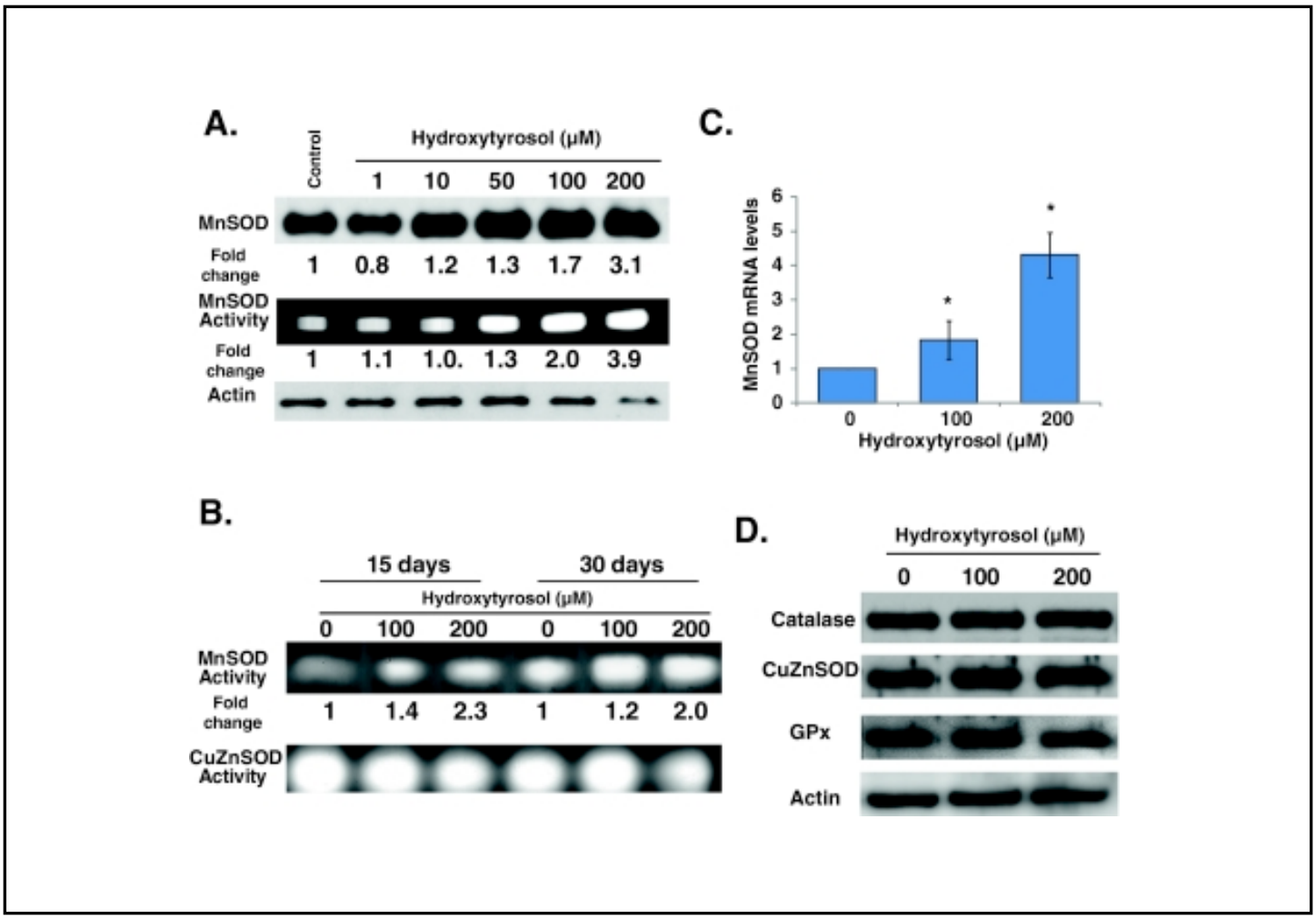

Figure 4: Hydroxytyrosol increases MnSOD activity in quiescent NHFs. Immunoblotting and gel-electrophoresis assays for MnSOD protein levels and activity in quiescent NHFs that were incubated with HT for **a** 3, and **b** 15 and 30 days. Monolayer quiescent cultures of NHFs were fed with HT-containing fresh media every 3 days. Actin protein levels were used for loading correction. Sodium cyanide was used to distinguish between MnSOD and CuZnSOD activities. **c** Quantitative RT-PCR assay measuring MnSOD mRNA levels in 3-day control and HT-treated quiescent NHFs; fold change was calculated relative to 18S and untreated control. *Asterisks* indicate significant difference between control and HT-treated cells. $n = 3$; $p < 0.05$. **d** Immunoblot analysis of antioxidant enzymes at the end of 3-day HT-treated quiescent NHFs.

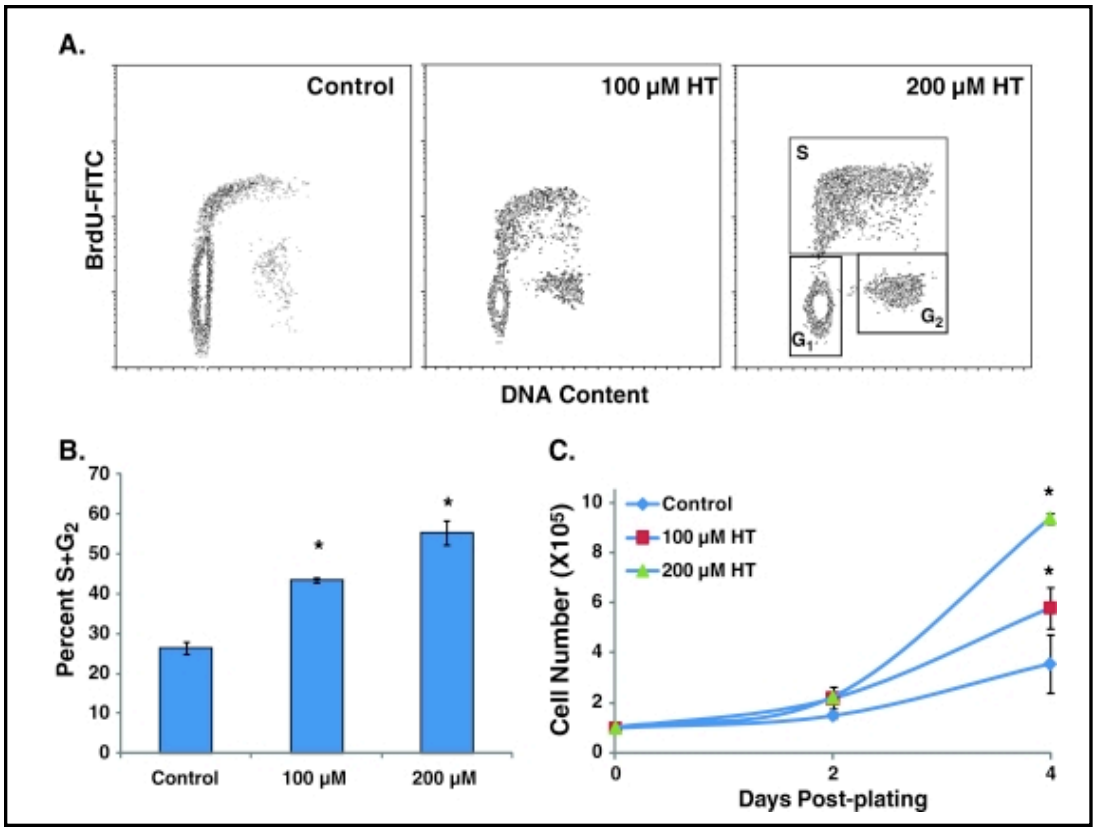

Figure 5: Hydroxytyrosol extends chronological lifespan after long duration of quiescence. Sixty-day control and HT-treated quiescent cultures of NHFs were replated at a lower cell density and cell growth characteristics were measured by flow cytometry analysis of BrdU-labeled cells at 48-h post-plating: **a** representative BrdU vs. PI density plot of DNA content; **b** percentage of S and G2 (proliferative) phases were evaluated using FlowJo and ModFit software; **c** cell numbers at 2- and 4-day post-plating. *Asterisks* indicate significant difference compared with untreated control. $n = 3$; $p < 0.05$

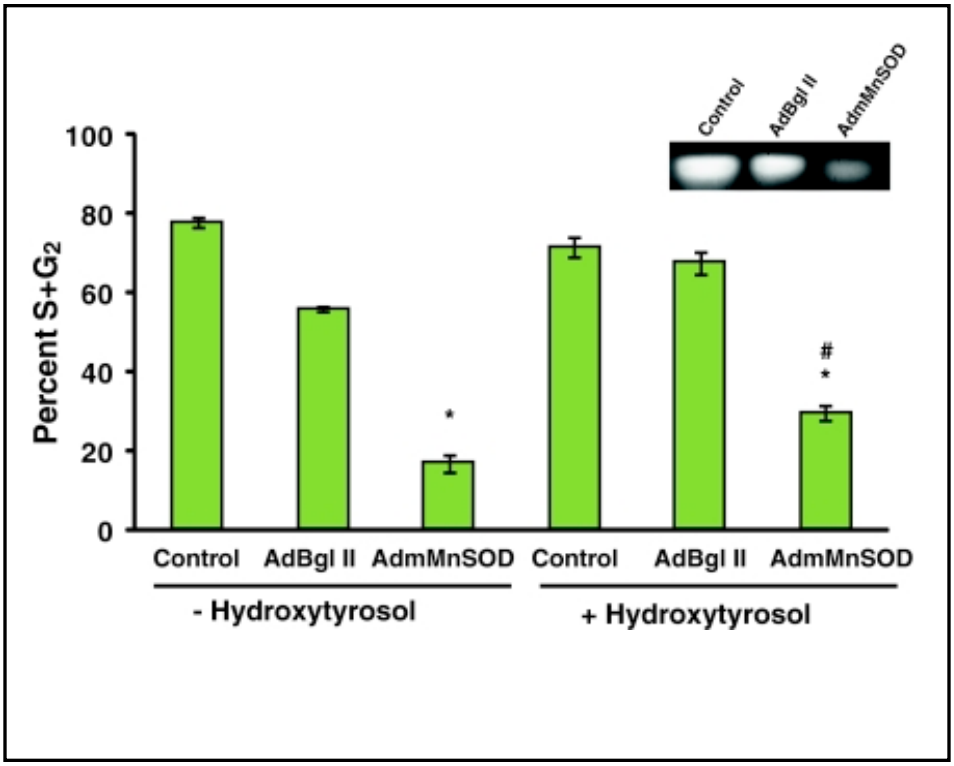

Figure 6: MnSOD activity regulates hydroxytyrosol-induced extension of chronological lifespan. Quiescent NHFs were infected with 50 MOI of adenoviruses carrying a control vector (AdBgl II) or a dominant-negative mutant form of human MnSOD cDNA (AdmMnSOD). Forty-eight hours post-infection cells were incubated with 200 μM HT for 3 days. Uninfected control and infected cells were replated at a lower cell density and harvested at 24-h post-plating for flow cytometry analysis of DNA content. MnSOD activity was analyzed using native gel-electrophoresis assay. *Asterisks* indicate significant difference in AdmMnSOD-infected cells compared with control; *number sign* represents statistical significance between HT-treated AdmMnSOD-infected cells compared with AdmMnSOD-infected cells. $n = 3$; $p < 0.05$

Since it was known that MnSOD protein protects quiescent normal human fibroblasts (NHF) derived from skin, against age-associated loss in proliferative capacity, the possibility that hydroxytyrosol preserves the chronological lifespan of quiescent fibroblasts was also investigated.[2] Hydroxytyrosol-induced extension of chronological lifespan was associated with an approximately 3-fold increase in MnSOD activity.Recently, it has been shown that ROS can function as secondary messengers in numerous signaling pathways that regulate several cellular processes including cellular proliferation.[9] N-acetyl-L-cysteine (NAC), a powerful antioxidant used to treat toxicity-related health issues, is also frequently used in research to study and manipulate a wide variety of oxidation-reduction (redox)-sensitive signaling processes. In particular, NAC is known to induce ROS signaling. Cellular proliferation is a highly coordinated event requiring sequential assembly and activation of protein complexes

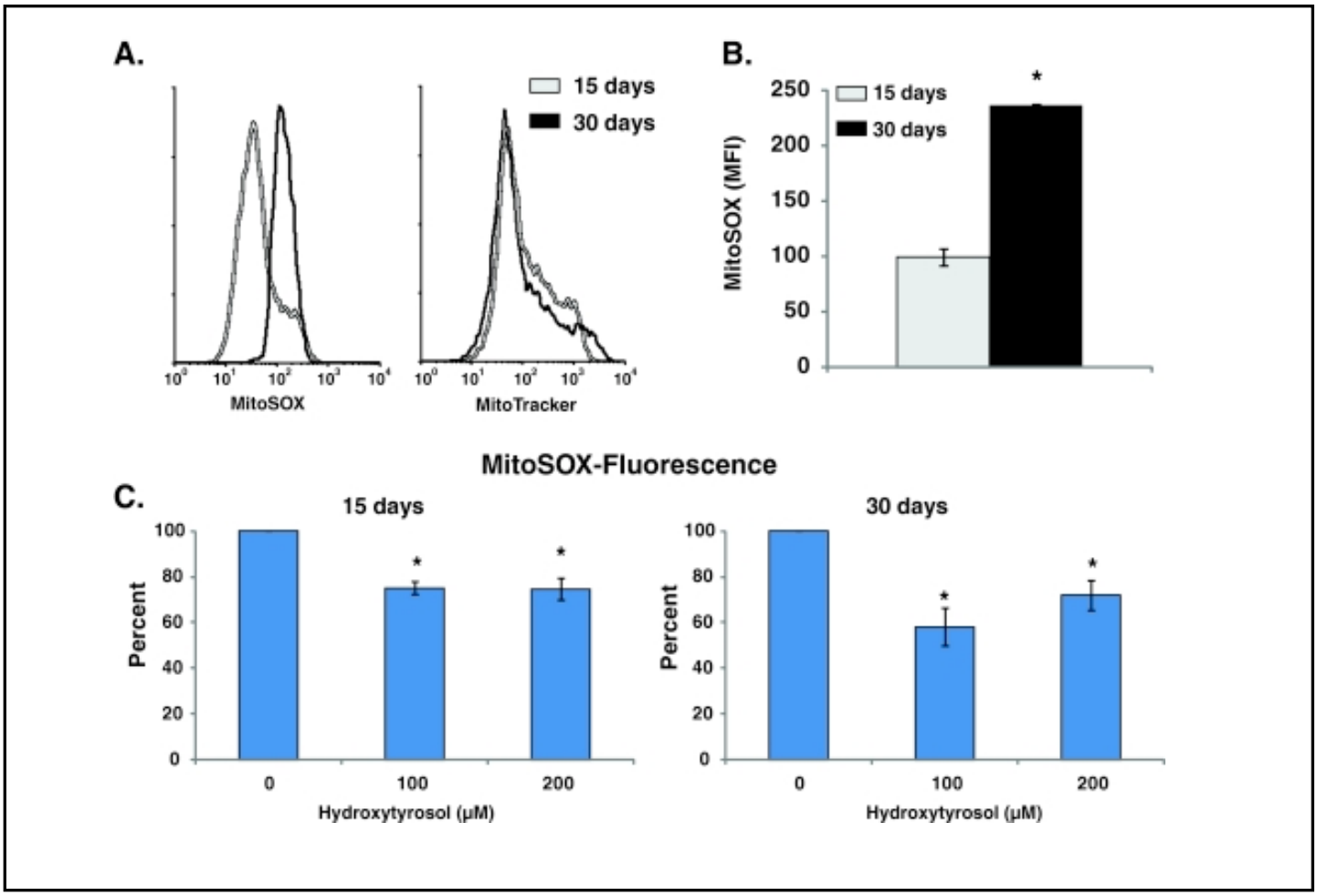

Figure 7: Hydroxytyrosol suppresses age-associated accumulation of mitochondrial ROS during quiescence. Control and HT-treated 15- and 30-day quiescent NHFs were incubated with 10 μM MitoSOX and 0.5 μM MitoTracker green and fluorescence measured by flow cytometry following our previously published method.[3] MitoSOX fluorescence was normalized to MitoTracker green fluorescence in each sample and percent change calculated relative to untreated control. **a** Representative histograms of MitoSox and MitoTracker in 15- and 30-day quiescent NHFs; **b** percent change in MitoSox fluorescence in 30- compared with 15-day untreated quiescent NHFs; **c** percent change in MitoSox fluorescence in HT-treated 15- and 30-day quiescent NHFs relative to untreated control. Asterisks indicate significant difference between HT-treated and untreated NHFs; $n = 3$, $p < 0.05$

that include the cyclin proteins. It was hypothesized that NAC affects cellular proliferation by regulating the cell cycle regulatory protein, cyclin D1 and by activating MnSOD. NAC was shown to regulate cell cycle progression by decreasing expression levels of cyclin D1 protein and by increasing expression and activity levels of MnSOD (similar to hydroxytyrosol).

These results demonstrate that at least some of NACs antioxidant properties may be due to its ability to increase MnSOD activity. The increase in MnSOD activity induced by NAC also suggests that NAC may protect cells from aging in a similar manner to hydroxytyrosol.

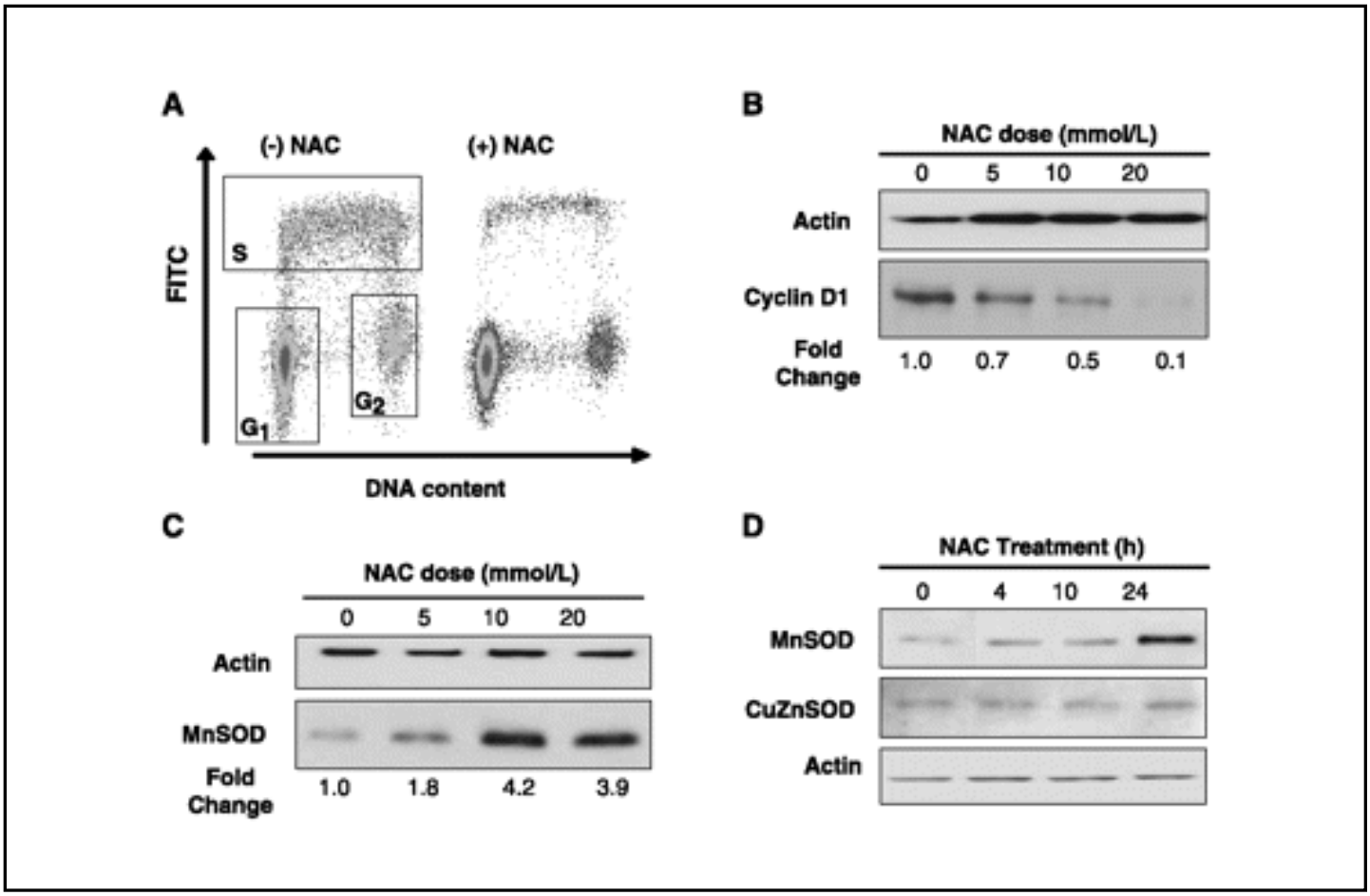

Figure 8: NAC-induced decreased cyclin D1 and increased MnSOD protein levels. (A), exponentially growing NIH3T3 fibroblasts were treated with 0, 5, 10, and 20 mmol/L NAC for 24 h. Cell cycle phase distribution was measured using BrdUrd pulse and dual variable flow cytometry assay. Representative FITC-PI histograms of cell cycle phase distributions in control and 24 h after exposure to 20 mmol/L NAC. Western blot analysis of cyclin D1 (*B*) and MnSOD (*C*) protein levels after 24 h of treatment with NAC. (D) immunoblot analysis of MnSOD protein levels from lysates treated with 20 mmol/L NAC for 4, 10, and 24 h.

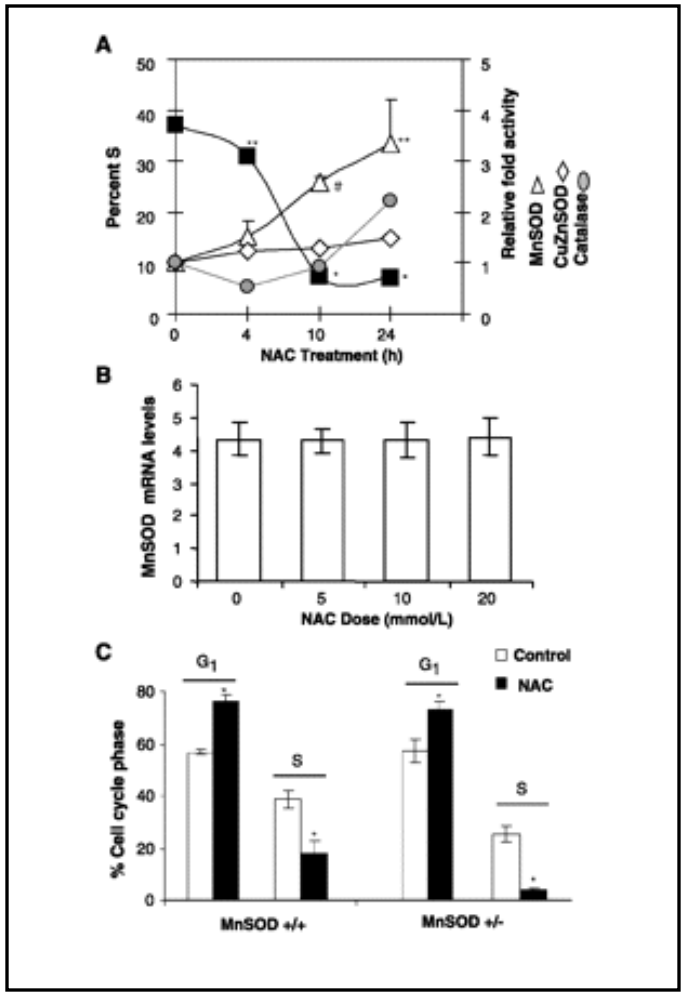

Figure 9: NAC-induced MnSOD activity aides in regulating cell proliferation. *A,* exponentially growing 3T3 fibroblast cells treated with 20 mmol/L NAC were collected at different intervals and assayed for enzymatic activity of MnSOD, CuZnSOD, and catalase. Relative activity of each enzyme plotted and compared with changes in percentage of S phase. *, $P < 0.001$; **, $P < 0.01$; #, $P < 0.05$ versus 0 h. *B,* real-time quantitative PCR analysis of *MnSOD* mRNA levels from samples treated with different doses of NAC (0–20mmol/L) for 24 h. 18S mRNA levels were used for internal control. Quantitation of MnSOD mRNA was done by normalizing to 18S mRNA levels in individual samples; $n = 3$. *C,* mouse fibroblasts with wild-type and heterozygous MnSOD were exposed to 20 mmol/L NAC for 24 h and collected for PI staining and flow cytometry analysis of cell cycle phase distribution.

In Summary, MnSOD activity was found to protect mitochondria from age-associated damage, and to preserve the chronological lifespan of quiescent cells. The regulatory role of mitochondrial ROS in cellular aging suggested that the oxygen environment was critical in regulating the chronological lifespan of the cells. It was discovered that the lower oxygen (4%) environment protected quiescent cells from age-associated abnormalities and loss of proliferative capacity. The anti-aging activities of hydroxytyrosol were also investigated in terms of its proposed activation of MnSOD activity. Hydroxytyrosol significantly extended the lifespan of quiescent fibroblasts, and this was associated with an approximately 3-fold increase in MnSOD activity. It was also discovered that MnSOD activity regulated the hydroxytyrosol-induced extension of chronological lifespan. ROS signaling is involved in the regulation of cell proliferation. The possibility that the antioxidant, N-acetyl-L-cysteine (NAC), which is known to induce ROS signaling, could also influence cellular proliferation was investigated. NAC was discovered to activate MnSOD and affect cellular proliferation by regulating the cell cycle regulatory protein, cyclin D1. The increase in MnSOD activity induced by NAC suggests that NAC may protect cells from aging in a similar manner to hydroxytyrosol. In addition, it is anticipated that NAC and hydroxytyrosol could be aided by the addition of key amino acids and cofactors associated with the repair process.

Acknowledgements

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