MANGANESE SUPEROXIDE DISMUTASE PROTECTS THE PROLIFERATIVE CAPACITY OF CONFLUENT NORMAL HUMAN FIBROBLASTS

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We tested the hypothesis that manganese superoxide dismutase (MnSOD), an antioxidant enzyme, regulates the proliferative potential of confluent human fibroblasts. Normal human skin (NHF: AG01522) and lung (WI38: CCL-74) fibroblasts kept in confluence (>95% G0/G1) showed a significant decrease in their capacity to re-enter the proliferation cycle after 40-60 days. The inhibition of re-entry was accompanied with the confluent culture’s age-dependent increase in p16 protein levels. Adenoviral mediated overexpression of MnSOD during confluent growth suppressed p16, enhanced p21 protein accumulation, and protected fibroblasts against the loss of proliferation potential. Increases in p21 protein levels in MnSOD overexpressing confluent fibroblasts were independent of p53 protein levels. p53 protein levels did not change in control, AdBgl II, or AdMnSOD infected confluent cells cultured for 20 and 60 days. In addition, MnSOD-induced protection of confluent fibroblasts’ proliferation capacity was independent of their telomerase activity. However, telomerase transformed fibroblasts showed increased MnSOD expression in confluent growth, which maintained confluent fibroblasts’ capacity to re-enter the proliferation cycle. While inactivation of the retinoblastoma protein in cells sub-cultured from the 60-day confluent control, AdBgl II, and AdMnSOD infected fibroblasts was identical, only MnSOD overexpressing cells showed a higher percentage of S-phase. These results support the hypothesis that a redox-sensitive checkpoint regulates fibroblasts’ progression from G0/G1 to S-phase.

In mammalian cells, intracellular antioxidant enzymes include superoxide dismutase (SOD), catalase, and glutathione peroxidase. There are two intracellular forms of SOD: CuZnSOD found in the cytoplasm and nucleus, and MnSOD in mitochondria (1, 2). Different isozymes of glutathione peroxidase are found in most subcellular compartments, while catalase is found primarily in peroxisomes and cytoplasm (1). Antioxidant enzymes neutralize reactive oxygen species (ROS) generated from the univalent reduction of oxygen by mitochondrial electron transport chains as well as biochemical reactions of oxygen metabolizing enzymes (3-5). ROS, including superoxide, hydrogen peroxide, hydroxyl radical, singlet molecular oxygen, and organic hydroperoxides are oxygen-containing molecules that have higher chemical reactivity than ground state molecular oxygen. ROS have traditionally been thought of as unwanted and toxic byproducts of living in an aerobic environment (6, 7). In recent years, several studies...
suggest metabolic production of ROS is tightly regulated and serves a physiological function during mitogenic stimulation of cultured cells (6-11). It has been suggested that ROS operate as a key signaling process in the cascade of events leading to cell proliferation following stimulation with platelet-derived growth factor (9), EGF (10), cytokines and antigen receptors (11), and oncogenic Ras (12). While these studies provide evidence for a possible role of redox signaling during cellular proliferation, it is currently unknown whether antioxidant enzymes regulate progression through the cell cycle.

Cell cycle is a tightly regulated sequence of transitions from G0/G1 to S to G2 to M phases, which requires assembly and activation of phase specific cyclin and cyclin-dependant kinase (Cdk) complexes (13-16). Cyclins D (D1, D2, and D3) and E in association with Cdns (Cdk2, Cdk4, and Cdk6) regulate progression from G1 to S. The enzymatic activity of cyclin/Cdk is regulated at three levels (15): cyclin binding and activation, subunit phosphorylation, and inhibition by one of the Cdk inhibitors [CIP/KIP family (p21, p27, and p57) and INK4 family (p15, p16, p18, and p19)]. Active cyclin/CDK kinase complex partially phosphorylates the retinoblastoma (Rb) protein, which causes the release of the E2F family of proteins initiating transcription of E2F-mediated gene expression and entry into S phase (17). While redox regulation of cell cycle regulatory proteins is not completely understood, other laboratories, including our own, have demonstrated that thiol-antioxidants affect cyclin D1, p21, p27, and Rb phosphorylation (18-20). These results support the hypothesis that intracellular redox environment could regulate the proliferative capacity of normal fibroblasts in culture.

Normal human fibroblasts (NHF) have a finite proliferative lifespan in culture prior to undergoing irreversible growth arrest, commonly known as the “Hayflick Limit” (21). In recent years, the majority of studies favor the idea that the Hayflick limit depends on the cumulative number of cell divisions rather than metabolic or calendar time. Therefore, a mitotic counting mechanism, “telomere attrition,” has been hypothesized to be the molecular clock of cellular aging (22, 23). In addition to telomere attrition, two Cdk inhibitors, p16 and p21, mediate irreversible growth arrest in replicating cells (24-26). However, several other studies have shown confluent human fibroblasts kept for a prolonged period in culture lose their capacity to re-enter the proliferative cycle (27-29). Interestingly, increases in protein levels of both Cdk inhibitors p16 and p21 correlate with confluent cultures’ inability to re-enter the proliferative cycle. These previous reports clearly suggest mechanisms other than cumulative cell division and telomeric attrition could regulate proliferative capacity of human normal fibroblasts. We therefore tested the hypothesis that MnSOD could regulate the proliferative capacity of confluent normal human diploid fibroblasts.

Materials and methods

Cell Culture - Human normal skin (NHF: AG01522) and lung (WI38: CCL-74) fibroblasts were obtained from Coriell Cell Repositories and ATCC, respectively. Generation of telomerase immortalized NHF (NHF+hTERT) has been described previously (30). All cell lines were maintained in DMEM medium with 10% fetal bovine serum, supplemented with penicillin-streptomycin antibiotics. All experiments were performed using cells from passage 8.

Adenovirus infections - Confluent cultures were infected with replication deficient adenovirus containing cytomegalovirus promoter driven human MnSOD cDNA containing the N-terminal mitochondrial localization signal (AdMnSOD). The MnSOD cDNA was inserted into the E1 region of an E1/partial E3-deleted replication deficient adenoviral vector (31). A non-modified vector (AdBgl II, ref. 32-34) was used as control for adenoviral infections. All adenovirus constructs were obtained from the University of Iowa DNA-Vector core. Transduction efficiency was measured by flow cytometry assay of AdGFP infected confluent cultures. MnSOD expression
was measured by immunoblotting, and a gel-electrophoresis based assay was used to measure MnSOD enzyme activity (35).

**Confluent cultures’ proliferative capacity assay** - Initially, one million cells per T-75 tissue culture flask were plated for each cell line and cultured in regular medium until confluence. Cells from replicate flasks were trypsinized and assayed for cell cycle phase distributions by flow cytometry measurements of DNA content and cell number. In general, cells reached confluence after 7 days of plating. Cells with less than 5 percent S-phase were considered a confluent culture; this marked day 1 of their experimental duration. Adenoviral infections were performed at day 1 of confluence using 30 MOI of either AdMnSOD or AdBgl II. Alternatively, infections were performed in cultures kept in confluent growth for 50 days. In either protocol, cells were kept in confluent growth for a total of 60 days and medium was replaced every 3 days. Control, AdBgl II-, and AdMnSOD-infected confluent cultures from 10, 20, 40, and 60 days confluence were subcultured by trypsinization and re-plated at a lower cell density (1:5 fold dilution). Re-plated cells were allowed to grow for 48 hours, and pulse-labeled with bromodeoxyuridine (BrdUrd) for 30 min prior to harvesting by trypsinization. Ethanol-fixed cells were analyzed for cell cycle phase distributions by flow cytometry. Changes in S-phase distributions were used as a measure of cells’ proliferative capacity (see below).

Cell population doubling times were calculated from growth curves. Re-plated cells were continued in culture and cell numbers counted up to 6 days. Cell population doubling time (Td) were calculated from the exponential portion of the growth curve using the following equation: Td = \(0.693t \ln \left( \frac{N_t}{N_0} \right)\) where t is time in days, Nt and N0 represent cell numbers at time t and the initial time.

**Flow cytometry assays** - Flow cytometry assays for measurements of cell cycle phase distributions were performed following a previously published protocol (18). Briefly, nuclei isolated from ethanol-fixed cells were incubated with anti-BrdUrd primary antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) followed by incubation with FITC-conjugated goat anti-mouse IgG. Nuclei were treated with RNase A (0.1 mg/ml), and counterstained with 20 µg/ml PI. FITC and PI fluorescence were analyzed on a Becton Dickinson FACScan flow cytometer. Data from a minimum of 20,000 nuclei were acquired and were processed using CellQuest Pro software (Becton Dickinson Immunocytometry Systems, San Jose, CA). The acquired data were analyzed as dual-parameter propidium iodide (PI) versus log-FITC histograms and three compartments (BrdUrd-positive S-phase cells, BrdUrd-negative G1 and G2 phases) were identified. The number of cells in each compartment were calculated and expressed as percentage of total gated population.

For univariate flow cytometry assay of DNA content, ethanol fixed cells were treated with RNase A for 30 min followed by staining with PI. PI-stained cells were analyzed by flow cytometry, and cell cycle phase distributions were calculated using CellQuest Pro and ModFit softwares (Verity Software House, Topsham, ME).

PI staining of unfixed cells was used to measure viability. Monolayer cultures were trypsinized, and re-suspended in 0.5 ml PBS containing PI prior to flow cytometry. Percentage of PI-positive (non-viable) and negative (viable) cells was determined using WinMD software.

**Immunogold staining and electron microscopy** - Control and adenovirus infected cultures were collected by scraping monolayer cells 5 days post-infection, and fixed with a light fixative mixture of 4% paraformaldehyde and 0.05% gluteraldehyde. Immunogold-staining was performed at the University of Iowa Central Microscopy Research Core Facility using MnSOD rabbit anti human polyclonal antibodies and F(ab)2 goat anti-rabbit ultra-small gold conjugated secondary antibodies.

**Immunoblotting assay** - Equal amounts of total protein extracts were separated by 12.5% or 7% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and probed with primary
antibodies against human MnSOD, CuZnSOD, p53, p21, and p16. MnSOD and CuZnSOD rabbit anti-human polyclonal antibodies were obtained from the Radiation and Free Radical Research Core Facility (The University of Iowa); p53 (PharMingen, BD, San Diego, CA), p21 (Upstate Biotechnology, Lake Placid, NY), p16 (PharMingen BD, San Diego, CA) and actin (Chemicon International, Temecula, CA) antibodies were used according to the manufacturer’s supplied protocol. Immunoreactive bands were detected by an enhanced chemiluminescence plus kit from Amersham (ECL-plus). Bands were quantified with a computerized digital imaging system interfaced with Scion Image software (Scion Corporation, Frederick, MD). The integrated density value was obtained by integrating the entire pixel values in the area of one band after correction for background. All blots were re-probed with antibody to actin, and actin protein levels were used for loading corrections. Blots are representative of at least two separate experiments.

**MnSOD enzyme activity assay** - Fifty micrograms of total protein extracts were separated by native polyacrylamide gel electrophoresis following a previously published protocol (35). Gels were stained with nitroblue tetrazolium (NBT) and riboflavin-TEMED solution for 20 min at room temperature. CuZnSOD and MnSOD were differentiated by the addition of sodium cyanide in the staining solution (36). The bands were visualized and quantified with a computerized digital imaging system interfaced with Alphalmager 2000 software (Alpha Innotech, San Leandro, CA). Results are representative of two or more experiments.

**Telomerase activity assay** - Cells were resuspended in lysis buffer (0.01% NP-40, 10 mM Tris pH7.5, 50 mM KCl, 5 mM MgCl2, 2 mM dithiothreitool, 20% glycerol and protease inhibitors). Lysates were centrifuged for 30 min at 16,000g and supernatants used for immunoprecipitation. The hTERT proteins were immunoprecipitated with anti-hTERT antibodies and protein A/G Sepharose beads. Telomerase activity in the immunoprecipitates was determined using the Telomerase PCR–ELISA kit (Boehringer Mannheim, Mannheim, Germany) following a previously published protocol (30). Telomerase activity in HeLa cells was used as positive control for the assay. Protein extracts denatured at 95°C for 5 min were included as negative control. Changes in telomerase activities were calculated relative to the negative control.

**Statistical analysis** - Statistical analysis was done using the one and two-way analysis of variance (ANOVA) with Tukey’s honestly significant difference test. Homogeneity of variance was assumed with 95% confidence interval level. Results with P < 0.05 were considered significant.

**Results**

MnSOD overexpression protects confluent normal fibroblasts’ capacity to re-enter the proliferation cycle—We have used adenovirus mediated gene transfer methodology to determine whether MnSOD antioxidant enzyme protects normal human fibroblasts’ proliferative capacity. Confluent cultures of NHF (>95% G0/G1 cells, based on DNA content as determined by flow cytometry) were infected with 10-100 MOI of AdMnSOD or AdBgl II and harvested for MnSOD protein and enzyme activity measurements beginning at 48 hours post-infection (Figure 1). MnSOD protein levels increased in control cells 7 days post-plating compared to 48 hours post-plating (Figure 1A). This result is consistent with an earlier report in the literature demonstrating increased MnSOD protein and activity in confluent growth-arrested cells compared to exponential cultures (37). Both 10 and 30 MOI AdMnSOD infected cells showed a dose-dependent increase in MnSOD protein and enzyme activity at 48 hours post-infection, which remained elevated even after 7 days of confluent growth (Figure 1 A & B). Cells infected with 100 MOI showed a decrease in MnSOD protein levels after 7 days of confluent growth (Figure 1A). This decrease in MnSOD protein was associated with a
decrease in cell viability as measured by PI-staining of unfixed cells and flow cytometry (data not shown) suggesting this higher dose could result in toxicity. Because the majority of our experiments extended up to 60 days, we determined whether ectopic expression of MnSOD continued for this prolonged period of time. Confluent cultures were infected with 30 MOI of AdBgl II or AdMnSOD and kept in confluent cultures for 60 days with regular change in growth medium. The basal levels of MnSOD protein and enzyme activity in control and AdBgl II infected cells showed marginal changes at 60 days of confluence compared to 20 days confluent cultures (Figure 1C). However, AdMnSOD infected cells continuously maintained 2-4 fold higher levels of MnSOD expression throughout the 60 days of confluent growth (Figure 1C). Similar results were also obtained for AdMnSOD infected confluent WI38 fibroblasts held in confluence for 60 days (Figure 5C). Furthermore, the duration of confluent growth did not show any significant changes in CuZnSOD protein levels in control, AdBgl II, and AdMnSOD infected cells (Figure 1C).

Next, we determined if overexpression of MnSOD in AdMnSOD infected cells caused MnSOD to be localized to the mitochondria. Immunologic detection of MnSOD using primary antibodies to MnSOD and ultra-small gold conjugated secondary antibodies show overexpression of MnSOD increased MnSOD-immunoreactive protein levels in the mitochondria of both 30 and 100 MOI AdMnSOD infected cells (Figure 2A). Flow cytometry measurement of GFP fluorescence in AdGFP infected cells was performed to determine transduction efficiency. Confluent cultures infected with 30 MOI AdGFP showed approximately 80 percent GFP-positive cells and the percentage of GFP-positive cells increased to approximately 96 percent in 100 MOI infected cells (Figure 2B). These results suggest the majority of cells express the transgene, and MnSOD protein levels were higher in the mitochondria of AdMnSOD infected cells compared to controls.

Confluent fibroblasts’ proliferative capacity was measured by sub-culturing confluent cultures at a lower cell density and monitoring BrdUrd incorporation 48 hours after re-plating. Confluent cultures of NHF were sub-cultured at 10, 30, 40, and 60 days of confluent growth, and re-plated at a lower cell density. Forty-eight hours later, cells were pulse-labeled with BrdUrd and ethanol fixed for flow cytometry measurements of cell cycle phase distributions. Results presented in Table 1 show more than 95% G0/G1 cells in each treatment group, suggesting the majority of cells were growth arrested prior to sub-culturing at both 20 days and 60 days of confluence. However, cell cycle phase distributions following 48 hours of re-plating were different among the three treatment groups. Figure 3A shows representative BrdUrd-PI bivariate histograms of cell cycle phase distributions at 48 hours post-plating of cells sub-cultured from 10 day (upper panel) and 60 day (lower panel) confluent cultures. NHF sub-cultured from 10 and 30 days confluent cultures showed approximately 20-25% S-phase cells in control, AdBgl II, and AdMnSOD infected cells (Figure 3B). However, control and AdBgl II infected NHF sub-cultured from confluent cultures held in confluence for 40-60 days showed a significant inhibition in re-entry to the proliferation cycle: 10-15% in control and 1-3% S-phase in AdBgl II infected cells (Figure 3B, Table 2). In contrast, cells sub-cultured from AdMnSOD infected confluent NHF showed a consistently higher percentage of S-phase cells, even after 60-days of confluent growth, approximately 20% in AdMnSOD infected cells compared to 2% S-phase cells in AdBgl II infected cells (p<0.05, Table 2).

MnSOD overexpression also prolonged confluent normal human lung fibroblasts (WI38) capacity to re-enter the cell cycle (Figure 3C). Similar to NHF, control and AdBgl II infected confluent WI38 fibroblasts lost their proliferation potential after 30 to 40 days in culture: 25% S-phase in cells sub-cultured from 10 days confluent culture compared to 4% S-phase in cells sub-cultured from 40-60 days confluent culture. In contrast, the percentage of S-phase cells in
MnSOD overexpressing WI38 fibroblasts remained essentially unaltered in cells sub-cultured from 60 days confluent culture compared to cells sub-cultured from 10 days confluent culture. These results clearly demonstrate a finite duration of the proliferative potential of human fibroblasts kept in confluent growth, and that MnSOD protects the proliferative capacity of confluent normal human fibroblasts.

MnSOD-dependent prolongation of NHF proliferation potential was also apparent from preservation of NHF growth characteristics, measured by counting cell numbers up to 6 days after sub-culturing (Figure 4A). The increase in cell numbers in cultures sub-cultured from 10-day confluent cultures did not show any significant difference among control, AdBgl II, and AdMnSOD infected cells (Figure 4A, left panel). However, cell growth in cultures sub-cultured from 60-day confluent control and AdBgl II infected NHF were significantly delayed (Figure 4A, right panel). These observations support the results from Figure 3 and clearly demonstrate NHF kept in confluent culture gradually lose their capacity to re-enter the cell cycle. Interestingly, MnSOD overexpression protected confluent NHFs’ capacity to re-enter the proliferation cycle even after 60 days in confluence.

Furthermore, subsequent to re-entry the cell number continued to increase during 6 days of post-sub-culturing (Figure 4A, right panel). To determine whether the inhibition in cell growth following re-plating of 60-day confluent cultures could be due to cell death and/or cell cycle arrest, DNA content was assayed at 2, 4, and 6 days post-plating. Ethanol-fixed cells were stained with PI and DNA content analyzed by flow cytometry. Results from these experiments showed a significantly higher percentage of cells with sub G0/G1 DNA content in control and AdBgl II infected cells compared to AdMnSOD infected cells (Figure 4B & C). The increase in percentage of cells with sub G0/G1 DNA content corresponded to a decrease in cell number in control and AdBgl II infected cells, suggesting the decrease could be due to cell death. Interestingly, while cell number at 2-day post-plating did show a decrease in control and AdBgl II compared to AdMnSOD infected cells, there were no change in cell population with sub G0/G1 DNA content. This difference in cell numbers at two days post-plating could be due to cell cycle arrest.

MnSOD overexpression suppresses accumulation of p16 and enhances p21 cyclin dependent kinase inhibitors protein levels during fibroblasts’ confluent growth—Because changes in p16 and p21 protein levels are associated with replicating fibroblasts’ irreversible growth arrest, we determined whether similar changes are also associated with confluent fibroblasts’ loss of proliferative capacity. Results presented in Figure 5 demonstrate increased p16 protein levels in control and AdBgl II infected confluent cultures at 60 days post-confluence both for NHF (Figure 5A) and WI38 (Figure 5C) cultures. In contrast, MnSOD overexpression suppressed p16 protein accumulation in both cell lines. MnSOD-induced decrease in p16 protein levels correlated with protection of fibroblasts’ proliferative capacity. Interestingly, while MnSOD overexpression suppressed p16 accumulation in cultures held at confluence for 60 days, the same treatment caused an increase in p21 protein levels both in NHF and WI38 fibroblasts (Figure 5). The increase in p21 protein levels was independent of changes in p53 protein levels. Results showed no change in p53 protein levels in confluent cultures of control, AdBgl II and AdMnSOD infected cells at 20 and 60 days of post-confluence (Figure 5B). This suggests MnSOD mediated protection of confluent fibroblasts’ proliferative capacity could be mediated via MnSOD-dependent regulation of the Cdk inhibitors, p16 and p21, protein levels.

We then determined whether MnSOD overexpression in cells that have already accumulated p16 protein could reverse the inhibition in cell cycle re-entry. NHF kept in confluence for 1 and 50 days were infected with 30 MOI AdMnSOD or AdBgl II and harvested after 60 days of initial confluent growth, i.e., confluent cultures infected on day 1 of confluence
were kept in confluent growth for 59 days while cells infected at 50 days of confluent growth were kept in confluence for an additional 10 days. Results presented in Figure 6 show that while AdMnSOD infection on day 1 of confluence suppresses p16 accumulation, MnSOD overexpression after 50 days of confluence had no significant effect on p16 protein levels (Figure 6A, lanes 4 and 7). Accordingly, confluent fibroblasts infected with AdMnSOD after 50 days of confluence showed no significant difference in S-phase distributions 48 hours after sub-culture compared to control and AdBgl II infected cells (Figure 6B, p>0.05). Thus, while MnSOD overexpression prior to increases in p16 protein levels can suppress p16 accumulation, MnSOD overexpression after p16 protein accumulation was ineffective in suppressing p16 protein accumulation.

Because telomerase activity has been shown to regulate exponential cultures’ replicative capacity, we determined whether MnSOD-dependent protection of confluent fibroblasts’ proliferation capacity could be due to changes in telomerase activity. Control, AdMnSOD, and AdBgl II infected confluent NHF kept in cultures for 60 days were assayed for telomerase activity (Figure 7). These results showed no significant difference in telomerase activity among control, AdBgl II, and AdMnSOD infected cells. These results suggest MnSOD dependent protection of confluent fibroblasts’ proliferation potential is independent of telomerase activity.

*Increased MnSOD protein and enzyme activity in confluent cultures of telomerase immortalized normal human fibroblast protected cells capacity to re-enter the proliferation cycle—* The relationship between MnSOD antioxidant enzyme activity in confluent cultures and protection of NHF’s proliferation capacity was further tested in telomerase immortalized NHF. Telomerase immortalization of NHF has been shown previously to increase MnSOD mRNA levels 6-7 fold (30). Initially, MnSOD protein levels and enzyme activity were measured in exponential growth and cells transiting to confluent growth stage (Figure 8A). Both NHF and telomerase immortalized NHF (NHF+hTERT) showed comparable MnSOD protein and enzyme activity during exponential growth (20-30% S-phase, based on flow cytometry measurement of DNA content). Although MnSOD protein levels and enzyme activity increased in both cell lines during cells’ transit to confluent growth stage (<10% S-phase), NHF+hTERT showed higher levels compared to NHF (lanes 3 and 6, Figure 8A). MnSOD protein levels continued to increase in confluent NHF+hTERT cultures at 10, 30, and 60 days of confluent growth (Figure 8B).

Increased MnSOD protein levels in confluent NHF+hTERT protected cells’ capacity to re-enter the proliferation cycle. Results demonstrate that while percentage of S-phase upon re-entry decreased in NHF sub-cultured from 60 days of confluent growth (<5% compared to 10% S-phase in cells sub-cultured from 20 days of confluent growth, Figure 8D), S-phase percentage remained high (>30%, Table 2) in NHF+hTERT sub-cultured from either 20 or 60 days of confluent growth. Because both cell lines had >95% G0/G1 cells prior to sub-culturing and only NHF+hTERT maintained high S-phase upon re-entry to the proliferation cycle, these results further show increased MnSOD levels in confluent cultures maintain fibroblasts’ reproductive integrity. Interestingly, hTERT-induced increase in MnSOD protein levels in confluent cultures was associated with inhibition in confluent fibroblasts’ age-dependent increase in p16 protein accumulation (lanes 3 and 6, Figure 8B). Increased MnSOD protein levels were also associated with enhanced p21 protein accumulation. Suppression of p16 accumulation in confluent NHF+hTERT fibroblasts facilitates cells’ entry into the proliferation cycle (Figure 8 C & D).

*MnSOD overexpression in confluent fibroblasts did not affect Rb-phosphorylation upon re-entry to the proliferation cycle—* Since hyper-phosphorylation of Rb accompanies cells’ entry from G1 to S, we determined whether the inability of 60-day confluent cultures to enter S-phase could be due to the differences in Rb-
phosphorylation. Sixty-day confluent cultures of control, AdBgl II, and AdMnSOD infected NHFs were sub-cultured and re-plated at a lower cell density. Re-plated cells were harvested 48 hours later and analyzed for p16, p21, and Rb-phosphorylation by immunoblotting. In 60-day confluent cells prior to sub-culturing, Rb was hypo-phosphorylated in control, AdBgl II, and AdMnSOD infected cells (lanes 1-3, Figure 9). As shown before (Figure 5), p16 protein levels in confluent fibroblasts were higher in control and AdBgl II infected cells compared to AdMnSOD infected cells, and overexpression of MnSOD enhanced p21 protein levels. Although p16 protein levels decreased substantially following re-entry into the proliferation cycle, AdMnSOD infected cells still showed higher p21 protein levels compared to control and AdBgl II infected fibroblasts (lanes 4, 5, and 6, Figure 9). Interestingly, inactivation of Rb following re-entry into the proliferation cycle was essentially similar in control, AdBgl II, and AdMnSOD infected cells (lanes 4-6, Figure 9). While Rb is hyper-phosphorylated in all three experimental conditions, only MnSOD overexpressing cells entered S-phase (Figure 3A) and continued to proliferate as evidenced from the increases in cell numbers (Figure 4A).

Discussion

Human somatic cells are known to have a finite number of proliferative lifespan prior to irreversible growth arrest. This phenomenon, commonly known as the “Hayflick Limit” (21) or replicative life span, is measured by counting the number of times a cell population undergoes cell division and doubles in size. While this concept is still considered a fundamental characteristic of cellular aging, another characteristic of aging appears to be the maintenance of reproductive integrity of G0/G1 quiescent cells, also known as chronological life span. Several earlier studies report human fibroblasts kept in confluent culture in vitro for a long period of time have a reduced replicative life span (27-29). These observations are further supported from findings that fibroblasts cultured from skins of older individuals undergo fewer cycles of cell division compared to fibroblasts isolated from younger individuals (38). Consistent with these observations, our results show normal human diploid fibroblasts kept in confluent cultures for prolonged period of time lose their capacity to proliferate upon re-entry to the cell cycle.

Replicative life span of in vitro cell culture and in vivo model organisms (yeast, Drosophila, and Caenorhabditis elegans) is believed to be regulated by oxidative stress, successive erosion of telomere length during replication, and cell cycle checkpoint pathways (6-17). Life span extensions in transgenic flies overexpressing superoxide dismutase antioxidant enzymes support the idea that antioxidants could influence an organism’s longevity (39-42). Transgenic flies overexpressing the cytosolic antioxidant enzyme, copper and zinc containing superoxide dismutase (CuZnSOD), showed a 33 to 48% increase in life span (40-42). Overexpression of the mitochondrial antioxidant enzyme, MnSOD, demonstrated a dose-dependent increases in life span (42). Consistent with oxidative stress mediated regulation of life span, cells cultured in vitro under chronic hypoxia (3% oxygen partial pressure) showed an increase in replicative life span (43, 44). In yeast, MnSOD overexpression shortened the replicative life span but prolonged the chronological life span (39, 45), suggesting antioxidant enzymes could regulate different pathways in replicative vs. chronological life span. Our results show constitutive overexpression of MnSOD antioxidant enzyme during confluent growth protected normal human diploid fibroblasts from time-dependent loss of proliferative capacity (Figure 3). These results support the hypothesis that mitochondrial generated ROS might influence the proliferative potential of quiescent fibroblasts under in vitro cell culture conditions.

Although MnSOD-dependent regulatory pathways are not clearly defined, a recent study reports overexpression of extracellular superoxide dismutase in human fibroblasts slows telomere
shortening (46). Alternatively, confluent growth arrested human fibroblasts stably overexpressing human telomerase enzyme enhanced MnSOD expression 6-7 fold (30). The successive decrease in telomere length during cell division, also known as the “end replication problem,” has been attributed to the inability of the DNA replication machinery to completely replicate the very end of the lagging strand (47). While telomeric attrition requires S-phase and subsequent cell divisions during the replicative life span, results presented in Figure 7 suggest the same pathway might not influence confluent fibroblasts’ proliferative capacity. Because a change in telomerase activity is routinely used as an indicator of “telomeric attrition,” telomerase activity was measured at the end of 60-day confluent growth in control, AdBgl II, and AdMnSOD infected fibroblasts. Results presented in Figure 7 display no significant difference in telomerase activity among control, AdBgl II, and AdMnSOD infected cells. This is also consistent with the observation that approximately 100% of the cells were in G1-phase (Table 1), and absence of a cycling population is expected to have very little effect on telomerase activity and subsequently telomere length. The idea that telomeric attrition requires extensive cell division rather than the calendar time has also been supported from a number of reports in the literature (27, 28, 48). Allsopp et al. (48) and Sitte et al. (28) both reported no significant level of telomere shortening in mid-lifespan fibroblasts kept in confluent culture for 7 and 10 weeks, respectively. A more recent report from Munro et al. (27) showed very little telomere attrition in human fibroblasts kept in culture for 10 months with intermittent confluence. These results further suggest that telomere attrition is exclusively a mitotic counting mechanism in replicating human fibroblasts.

While telomere attrition might not be a significant event during confluence, fibroblasts immortalized with hTERT showed a gradual increase in MnSOD protein levels and activity during confluence compared to control (Figure 8). Considering constitutive expression of hTERT in these stable transfectants, it is not clear why hTERT overexpression selectively activates MnSOD expression in confluent vs. exponential (20-30% S phase) cells (Figure 8 A & B). Interestingly, increased levels of MnSOD in confluent hTERT-overexpressing fibroblasts (NHF+hTERT) also showed higher percentage of S-phase upon re-entry into the proliferation cycle (Figure 8 C & D, and Table 2). Thus, increased MnSOD protein levels and enzyme activity in confluent fibroblasts, either due to AdMnSOD infection or hTERT overexpression, appear to maintain the proliferative potential of these cells upon re-entry to the cell cycle. These results, as well as those of others (27, 28), suggest a large number of confluent cells undergo telomere-independent irreversible growth arrest (measured by their inability to re-enter the proliferation cycle), and only a small subset of confluent cells retain their reproductive integrity to repopulate.

Re-entry and progression through the cell cycle is a tightly regulated sequence of transitions from G0/G1 to S to G2 to M phases involving the sequential activation of cyclin-dependent kinase (Cdk) activities that are regulated positively by cyclins and negatively by Cdk inhibitors. Increases in both p16 and p21 Cdk-inhibitors during an irreversible growth arrest are generally thought to be associated with accumulation of hypo-phosphorylated Rb (24). Hypo-phosphorylated Rb is known to sequester members of the E2F transcription factor family, and unavailability of E2F inhibits progression through the cell cycle. Consistent with these reports, our results also demonstrated hypo-phosphorylated Rb during fibroblasts’ confluent growth in control, AdBgl II, and AdMnSOD infected cells (Figure 9), suggesting overexpression of MnSOD in confluent cells does not affect Rb-phosphorylation. However, MnSOD overexpression did alter p16 and p21 protein accumulation patterns during confluence. While age-dependent increase in p16 protein accumulation in confluent fibroblasts is associated with cells’ inability to re-enter the cell cycle, MnSOD-dependent suppression in p16 protein accumulation maintained confluent cells
capacity to re-enter the proliferation cycle (Figures 5, 8, and Table 2). Because p16 is a specific inhibitor of the cyclin D1/Cdk4, 6 kinase complex and Rb is one of the substrates for this kinase activity, suppression of p16 would be expected to facilitate Rb-phosphorylation upon re-entry to the proliferation cycle. Indeed, MnSOD overexpression-induced suppression of p16 in confluent fibroblasts is associated with Rb-hyperphosphorylation upon re-entry to the proliferation cycle and fibroblasts’ subsequent entry into S-phase (lane 6, Figure 9 and Figures 3 & 8). However, the Rb-phosphorylation pattern in MnSOD overexpressing cells following re-entry was not different compared to control and AdBgl II infected cells (lanes 4-6, Figure 9), yet S-phase distributions were much higher in MnSOD overexpressing cells. These results suggest MnSOD-induced decrease in p16 protein accumulation in confluent cells might have a different endpoint compared to p16’s role in Rb-phosphorylation in proliferating cells. Furthermore, an earlier report of p16 accumulation as a function of replicative senescence in mouse fibroblasts and p16 accumulation being independent of Rb gene status (26) further supports the idea that p16 could have a differential regulatory role in confluent vs. exponential growth phase. Alternatively, MnSOD-overexpression in confluent fibroblasts’ could preserve cell cycle re-entry pathways that are Rb-independent. Furthermore, because p16 protein levels decreased upon re-entry in control, AdBgl II, and AdMnSOD infected cells (Figure 9), with only AdMnSOD infected cells continuing to cycle upon re-entry, p16’s regulatory role during confluency is more critical in maintaining the reproductive integrity of confluent cultures. Consistent with this hypothesis, overexpression of MnSOD failed to protect the loss of proliferative capacity in confluent fibroblasts after p16 accumulated (Figure 5).

Interestingly, while MnSOD overexpression suppressed p16 protein accumulation in confluent fibroblasts, the same treatment enhanced p21 protein levels (Figure 5). MnSOD-dependent accumulation of p21 protein levels was independent of p53 protein levels (Figure 5B). While p16 is elevated at the end of replicative life span and drives cells to an irreversible growth arrest, increases in p21 protein levels are biphasic and p21’s inhibitory effect on cell growth is a reversible process. Recent discoveries suggest p21 has multiple functions including assembling cyclin/Cdk kinase complexes, inhibiting cyclin/Cdk kinase activity, binding to PCNA and regulation of DNA synthesis, association with ASK1 and possible regulation of cell death pathways, and binding to Rho protein and subsequent effects on integrin-signaling pathways (49). In addition, p21 has been shown to maintain hematopoietic stem cell quiescence, and consequently transplanted bone marrow from p21-knock out mice succumbed to hematopoietic failure (50). Furthermore, antisense p21 is known to release human mesenchymal cells from G0 (51). These reports support a regulatory role of p21 in maintaining quiescence and protecting the replicative integrity of proliferation-competent cells. It is currently unknown if any, or all of these p21 functions are sensitive to intracellular redox environment. However, our results suggest MnSOD induced protection of confluent fibroblasts reproductive integrity could be mediated via maintaining a higher p21 protein levels (Figure 5). It is possible that MnSOD-induced accumulation of p21 protein levels would protect cyclin E(A)/Cdk 2 kinase complexes, which upon sub-culturing would facilitate a Rb-phosphorylation independent entry into S-phase. Such an assumption is supported by previous studies in the literature demonstrating cyclin E dependent and Rb-phosphorylation independent entry into S-phase (24, 52). Nonetheless, the observation that MnSOD induces p21 protein levels might be of broad interest because of the possibility that antioxidants, in particular MnSOD, could have a protective role in maintaining the reproductive integrity of a variety of proliferation-competent quiescent cells, including stem cells.

In summary, our results demonstrate a balance between two critical Cdk inhibitors, p16 and p21, could mediate an MnSOD-induced
protection of confluent fibroblasts’ proliferation capacity (Figure 10). While a majority of aging research in mammalian cells focuses on understanding the mechanisms regulating the somatic cell’s replicative life span, very few studies are centered on the investigation of the mechanisms regulating the proliferative potential of quiescent cells, also known as chronological life span. Because longevity of an organism depends on proliferative capacities of cycling and replicative-competent non-cycling cells, a detailed understanding of the mechanisms regulating the chronological life span becomes highly essential. Our results support the hypothesis that two separate but interdependent pathways could regulate cellular longevity: a redox-sensitive checkpoint regulating transition from G0/G1 to S phase followed by telomeric attrition controlling the “mitotic clock.”

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The abbreviations used are: AdMnSOD: Replication defective adenovirus containing cytomegalovirus-promoter driven human Mn-superoxide dismutase antioxidant enzyme cDNA; AdBgl II: Replication defective adenovirus containing an insertless vector; BrdUrd: bromodeoxyuridine; CuZnSOD: copper and zinc-containing superoxide dismutase; FITC: fluorescence isothiocyanate; hTERT: human telomerase reverse transcriptase; MOI: multiplicity of infection; NHF: normal human fibroblasts; PI: propidium iodide; redox: reduction and oxidation; ROS: reactive oxygen species.
Figure legends

**Figure 1.** Expression of MnSOD antioxidant enzyme in confluent NHF: confluent NHF were infected (10-30 MOI) with AdMnSOD or AdBgl II, and harvested at 48 hours and 7 days post-infection for (A) immunoblotting of MnSOD protein levels and (B) gel-electrophoresis based MnSOD enzyme activity assay; (C) MnSOD protein levels and enzyme activities in 30 MOI AdMnSOD or AdBgl II infected NHF kept in confluent growth for 20, 40 and 60 days. Blots were re-hybridized for CuZnSOD and actin protein levels.

**Figure 2.** MnSOD localization and transduction efficiency: (A) electron microscopy visualization of mitochondria localized MnSOD; confluent NHF infected with AdBgl II and AdMnSOD were scrape-harvested 7 days post-infection, fixed, and immunostained with primary antibodies to MnSOD and immunogold-conjugated secondary antibody. Negative pictures were recorded at 30,000X magnification; bars represent 200 nm; insets represent sections of mitochondria showing immunogold-positive MnSOD protein within the mitochondria. (B) Flow cytometry assay for transduction efficiency: control and AdGFP infected (30 MOI and 100 MOI) confluent NHFs were kept in culture for 7 days, trypsinized and re-suspended in 500 µl PBS. GFP fluorescence measured by flow cytometry and GFP-positive and negative cell population analyzed by WINMDI software: FITC vs. forward scatter.

**Figure 3.** MnSOD overexpression protects G₀/G₁ cells’ capacity to re-enter S-phase. Confluent NHF were infected with 30 MOI of AdBgl II or AdMnSOD. Cells were kept in confluent growth with regular change in growth medium and sub-cultured by re-plating at lower cell densities beginning at 10 days of confluent growth. Re-plated cells were cultured for additional 48 hours and pulse-labeled with BrdUrd prior to harvesting. Cell cycle phase distributions were analyzed by flow cytometry following a previously published protocol (18). (A) Representative BrdUrd vs. PI bi-variate histograms of cell cycle phase distributions in cells sub-cultured from 10 and 60 days confluent growth; S-phase distributions in NHF (B) and WI38 (C) were calculated using CellQuest software n=5, * p<0.05. All cultures had > 95% G₁ cells (based on PI-staining and flow cytometry measurements of DNA content) prior to sub-culturing (Table 1).

**Figure 4.** MnSOD overexpression protects the proliferative capacity of confluent normal human fibroblasts. (A) Growth characteristics of control, 30 MOI AdBgl II, and AdMnSOD infected NHF sub-cultured from cultures kept at confluence for 10 (left panel) and 60 (right panel) days; n=3, * p<0.05. Cells from replicate plates were harvested by trypsinization and fixed in ethanol. Ethanol-fixed cells were treated with RNase A, stained with PI and DNA content analyzed by flow cytometry. Representative histograms are shown in (B), and fraction of cells with sub G₀/G₁ DNA content is shown in (C); an arrow marks sub G₀/G₁ peak.

**Figure 5.** MnSOD overexpression suppressed p16 and enhanced p21 Cdk inhibitors in confluent normal human skin and lung fibroblasts. Immunoblotting assay of cyclin dependent kinase inhibitors (p16 and p21) protein levels in control, 30 MOI AdBgl II, and AdMnSOD infected confluent cultures: (A) NHF, 20 and 60 days post-infection and (C) WI38, 60-days post-infection. (B) Immunoblot assay of p53 protein levels in 20 and 60-day confluent control, AdBgl II, and AdMnSOD infected NHFs. Actin protein levels were used for comparison of results.

**Figure 6.** MnSOD overexpression after p16 accumulation failed to reverse the inhibition in NHF re-entry into the proliferation cycle. Confluent NHF held at confluence for 50 days were infected with 50 MOI of AdMnSOD, or AdBgl II. Cells were continued in confluent culture for
10 days post-infection and harvested for immunoblot analysis (lanes 2-4, panel A). Cells from replicate plates were trypsinized and re-plated at a lower cell density. Forty-eight hours post-plating, cells were pulse-labeled with BrdUrd and assayed for S-phase distribution by flow cytometry (B), n=3, p>0.05. Lanes 5-7 in panel A represent proteins extracted from cells that were infected at day 1 of confluent growth and held at confluence for 60 days. Lane 1 represents cultures held at confluence for 50 days prior to the adenovirus infection.

**Figure 7.** MnSOD overexpression in confluent NHF cultures did not affect telomerase activity. Relative telomerase activity was measured by an *in vitro* PCR based ELISA assay; confluent cultures of NHF were infected with 30 MOI of AdBgl II, or AdMnSOD and continued in confluent cultures for 60 days. Telomerase assay was performed following a previously published protocol (30). (n=3, P>0.05)

**Figure 8.** Telomerase immortalized normal human fibroblasts showed increased MnSOD protein levels and enzyme activity during confluence, suppressed p16 and enhanced p21 protein levels, and maintained confluent fibroblasts’ capacity to re-enter the proliferation cycle. NHF and NHF immortalized with hTERT (NHF+hTERT) were harvested from (A) exponential (lanes 1, 2, 4 and 5), transit to confluent growth (lanes 3 and 6), and (B) 10, 30, and 60 days of confluent growth for immunoblotting analysis of MnSOD, p21 and p16 protein levels. Cells from 20, 30, 40, 50, and 60 days confluent growth were trypsinized and re-plated at lower cell density. Re-plated cells were cultured for additional 48 hours and pulse-labeled with BrdUrd prior to harvesting; (C) Representative BrdUrd vs. PI bi-variate histograms of cell cycle phase distributions in cells sub-cultured from 20 and 60 days confluent cultures; (D) S-phase distributions were calculated using CellQuest software. n=3, *p<0.05 at all time points for NHF compared to NHF+hTERT.

**Figure 9.** MnSOD overexpression induced protection of NHF’s proliferation capacity appears to be independent of Rb-phosphorylation status. Confluent NHF infected with 30 MOI AdBgl II or AdMnSOD were kept in confluence for 60 days and sub-cultured by re-plating at a lower cell density. Cells were collected prior to re-plating (lanes 1-3) and 48 hours after sub-culturing (lanes 4-6) for immunoblot analysis of p16, p21, and Rb. Actin protein levels were used for comparison of results. (Representative of blots from three different experiments).

**Figure 10.** A schematic illustration of MnSOD induced balance between p16 and p21 Cdk inhibitors, and confluent fibroblasts’ protection of proliferative capacity.
Table I: Cell cycle phase distributions in confluent NHFs

| Cell cycle phase | 20 Days | 60 Days |
|------------------|---------|---------|
|                  | Control | AdBgl II | AdMnSOD | Control | AdBgl II | AdMnSOD |
| G₁               | 98.8 ± 2.2 | 95.5 ± 0.3 | 96.7 ± 5.6 | 99.2 ± 0.3 | 97.9 ± 3.6 | 96.6 ± 0.3 |
| S                | 1.1 ± 1.9 | 4.5 ± 0.3 | 3.3 ± 5.6 | 0.4 ± 0.1 | 2.0 ± 3.6 | 2.1 ± 0.4 |
| G₂+M             | 0.1 ± 0.3 | 0        | 0        | 0.4 ± 0.4 | 0.8 ± 1.3 | 1.3 ± 0.0 |

Average ±SD; Flow cytometric measurement of DNA content, n=3, P>0.05
Table II: Flow cytometry measurements of cell cycle phase distributions at 48 hours post-plating of cells kept in confluent cultures for different days.

| Days in confluency | 10   | 40†  | 60‡  |
|--------------------|------|------|------|
|                    | G₁   | S    | G₂+M | G₁   | S    | G₂+M | G₁   | S    | G₂+M |
| NHF Control         | 61.2±8.0 | 21.9±5.8 | 17.0±4.1 | 84.2±12.8 | 8.7±7.6 | 7.1±5.6 | 80.8±10.0 | 7.7±4.3 | 11.6±5.9 |
| NHF AdBgl II       | 61.7±8.3 | 19.2±4.1 | 19.1±4.1 | 99.0±2.2 | 1.0±2.2 | 0.0±0.0 | 96.0±8.9 | 1.0±2.2 | 3.0±6.7 |
| NHF AdMnSOD        | 62.3±9.6 | 17.5±4.5 | 18.2±8.2 | 70.6±5.1 | 21.4±5.8 | 8.0±4.4 | 61.7±13.8 | 24.1±6.4 | 14.3±9.0 |
| NHF+hTERT          | 50.1±2.3 c | 37.0±0.4 c | 12.9±1.8 c | 50.7±1.1 | 24.3±0.6 | 25.0±1.7 | 55.5±2.6 | 30.4±2.1 | 14.2±0.6 |

average±SD; a, n=5; b, n=3; c, 20 days confluent cultures, † significant differences at all cell cycle distributions comparing NHF Control and AdBgl II to NHF AdMnSOD and NHF-hTERT (P<0.05).
Figure 1

A. Control AdBgl II (MOI) AdMnSOD (MOI)

|       | 30 | 10 | 30 | 100 |
|-------|----|----|----|-----|
| 48 h  |    |    |    |     |
| 7 d   |    |    |    |     |

B. Control AdBgl II (30 MOI) AdMnSOD (30 MOI)

|       | 48 h | 7 d  |
|-------|------|------|
| 1     |      |      |
| 2     |      |      |
| 3     |      |      |
| 4     |      |      |
| 5     |      |      |

C. Days in Confluency

| Days in Confluency | Control | AdBgl II | AdMnSOD |
|--------------------|---------|----------|---------|
| 20                 | 1.0     | 0.7      | 2.1     |
| 40                 | 1.2     | 0.8      | 2.1     |
| 60                 | 1.7     | 1.1      | 4.0     |

Fold MnSOD

|        | MnSOD | CuZnSOD | Actin | MnSOD activity |
|--------|-------|---------|-------|----------------|
| 1      |       |         |       |                |
| 2      |       |         |       |                |
| 3      |       |         |       |                |
| 4      |       |         |       |                |
| 5      |       |         |       |                |
| 6      |       |         |       |                |
| 7      |       |         |       |                |
| 8      |       |         |       |                |
| 9      |       |         |       |                |
Figure 2

A. 

Control | AdMnSOD 30 MOI | AdMnSOD 100 MOI | AdBgl II 100 MOI

B. 

Control | AdGFP 30 MOI | AdGFP 100 MOI

GFP + | 2% | 79% | 96%

GFP - | 98% | 21% | 4%
Figure 3

A.

Control    AdBgl II    AdMnSOD

Day 10

Day 60

B.

DNA Content

BrdUrd - FITC

C.

DNA Content

48 h post-plating

Days in Confluency

48 h post-plating

Days in Confluency

NHF Control
NHF AdBgl II
NHF AdMnSOD

WI-38 Control
WI-38 AdBgl II
WI-38 AdMnSOD

S phase (%)

S phase (%)

0 10 20 30

0 10 20 30

*
Figure 4

A.

DNA Content

| No. of cells |
|------------|
| Post-plating (d) |
| GG11         |

B.

60 days confluent

60 days confluent

Post-plating (d) 2 d 4 d 6 d

Sub G_0/G_1 (%)

Post-plating (d) 2 d 4 d 6 d

Control

AdBgl II

AdMnSOD

Sub G_0/G_1

*
Figure 5

A.

|                | NHF |      |      |
|----------------|-----|------|------|
|                | Day 20 | Day 60 |
| Control        |      |      |
| AdBgl II       |      |      |
| AdMnSOD        |      |      |

| p16            |      |      |
|----------------|------|------|
| p21            |      |      |
| Actin          |      |      |

B.

|                | NHF |      |      |
|----------------|-----|------|------|
|                | Day 20 | Day 60 |
| Control        |      |      |
| AdBgl II       |      |      |
| AdMnSOD        |      |      |

| p53            |      |      |
|----------------|------|------|
| p21            |      |      |
| Actin          |      |      |

C.

|                | WI-38 (Day 60) |
|----------------|----------------|
| Control        |                |
| AdBgl II       |                |
| AdMnSOD        |                |

| p16            |      |
|----------------|------|
| p21            |      |
| MnSOD          |      |
| Actin          |      |
### Figure 6

#### A. Adenovirus Transduction Schedule

|                  | 50 days after confluency | 1 day after confluency |
|------------------|--------------------------|------------------------|
|                  | Control                  | AdBgl II              |
| p16              |                          | AdMnSOD               |
| MnSOD            |                          |                        |
| Actin            |                          |                        |

#### B. S phase (%) over time

![Graph showing S phase (%) over time]

- **NHF Control**
- **NHF AdBgl II**
- **NHF AdMnSOD**

Post-plating (48 h)
Figure 7

60 days confluency

Relative Telomerase activity

Negative Control
Positive Control
NHF Control
NHF AdBgl II
NHF AdMnSOD
Figure 8

A.  

|          | NHF | NHF+hTERT |
|----------|-----|-----------|
| S Phase %| 21  | 14 6      |
| MnSOD    |     |           |
| Actin    |     |           |
| MnSOD Activity | 1 | 2 3 4 5 6 |

B.  

| Days in confluency | NHF | NHF+hTERT |
|--------------------|-----|-----------|
|                    | 10  | 30 60     |
| MnSOD              |     |           |
| p16                |     |           |
| p21                |     |           |
| Actin              |     |           |

C.  

Day 20  

Day 60  

NHF

NHF+hTERT

D.  

DNA Content

Day 20  

Day 60  

BrdUrd - FITC

S phase %

Post-plating (48 h)  

Days in confluency
Figure 9

NHF

|                | Confluent (day 60) | Post-plating (48 h) |
|----------------|--------------------|---------------------|
| Control        |                    |                     |
| AdBgl II       |                    |                     |
| AdMnSOD        |                    |                     |
| Control        |                    |                     |
| AdBgl II       |                    |                     |
| AdMnSOD        |                    |                     |

- **p16**
- **p21**
- **p-Rb**
- **Rb**
- **Actin**

Samples 1-6
Manganese superoxide dismutase protects the proliferative capacity of confluent normal human fibroblasts
Ehab H. Sarsour, Manjula Agarwal, Tej K. Pandita, Larry W. Oberley and Prabhat C. Goswami

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