Oxidative Stress Induces Premature Senescence by Stimulating Caveolin-1 Gene Transcription through p38 Mitogen-Activated Protein Kinase/Sp1–Mediated Activation of Two GC-Rich Promoter Elements

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Abstract

Cellular senescence is believed to represent a natural tumor suppressor mechanism. We have previously shown that up-regulation of caveolin-1 was required for oxidative stress–induced premature senescence in fibroblasts. However, the molecular mechanisms underlying caveolin-1 up-regulation in senescent cells remain unknown. Here, we show that subcytotoxic oxidative stress generated by hydrogen peroxide application promotes premature senescence and stimulates the activity of a (−1,296) caveolin-1 promoter reporter gene construct in fibroblasts. Functional deletion analysis mapped the oxidative stress response elements of the mouse caveolin-1 promoter to the sequences −244/−222 and −124/−101. The hydrogen peroxide–mediated activation of both Cav-1 (−244/−222) and Cav-1 (−124/−101) was prevented by the antioxidant quercetin. Combination of electrophoretic mobility shift studies, chromatin immunoprecipitation analysis, Sp1 overexpression experiments, as well as promoter mutagenesis identifies enhanced Sp1 binding to two GC-boxes at −238/−231 and −118/−106 as the core mechanism of oxidative stress–triggered caveolin-1 transactivation. In addition, signaling studies show p38 mitogen-activated protein kinase (MAPK) as the upstream regulator of Sp1-mediated activation of the caveolin-1 promoter following oxidative stress. Inhibition of p38 MAPK prevents the oxidant-induced Sp1-mediated up-regulation of caveolin-1 protein expression and development of premature senescence. Finally, we show that oxidative stress induces p38-mediated up-regulation of caveolin-1 and premature senescence in normal human mammary epithelial cells but not in MCF-7 breast cancer cells, which do not express caveolin-1 and undergo apoptosis. This study delineates for the first time the molecular mechanisms that modulate caveolin-1 gene transcription upon oxidative stress and brings new insights into the redox control of cellular senescence in both normal and cancer cells. (Cancer Res 2006; 66(22): 10805-14)

Introduction

Caveolae are invaginations of the plasma membrane enriched in cholesterol. Caveolin is the structural protein component of caveolar membranes. Caveolin acts as a scaffolding protein to concentrate and functionally regulate signaling molecules (1–7). The caveolin gene family consists of three members: caveolin-1, caveolin-2, and caveolin-3 (3, 4, 8). Caveolin-1 and caveolin-2 are coexpressed in many cell types, including adipocytes, endothelial cells, epithelial cells, and fibroblasts (9). In contrast, caveolin-3 expression is essentially restricted to skeletal and smooth muscle cells, as well as cardiac myocytes (10–18). The direct interaction with caveolin-1 results in the inhibition of a number of signaling molecules, such as G-protein α subunit, Ras, nitric oxide synthase, protein kinase C, and protein kinase A (2, 7, 10, 16–25). However, caveolin-1 has also been shown to stimulate the estrogen and insulin receptor signaling (26, 27).

Several independent lines of evidence indicate that caveolin-1 may act as an antiproliferative protein (28–32). Consistent with this idea, we have previously shown that overexpression of caveolin-1 is sufficient to arrest mouse embryonic fibroblasts in the G_0-G_1 phase of the cell cycle, reduce their proliferative life span, and promote premature cellular senescence through activation of a p53/p21–dependent pathway (33, 34).

According to the "free radical theory" of aging, normal aging occurs as the result of tissue damages inflicted by reactive oxygen species. In support to this theory, aged animals have been shown to produce higher levels of reactive oxygen species, compared with younger animals, due to defective mitochondria. In addition, increased oxidative damage of DNA, proteins, and lipids has been reported in aged animals (35). Thus, endogenous and exogenous stimuli may significantly increase oxidant levels within the cell and, as a consequence, induce a series of cellular damages. The molecular mechanisms that mediate the cellular response to oxidants remain to be fully identified.

Subcytotoxic oxidative stress is known to induce premature senescence in diploid fibroblasts. We have previously shown that subcytotoxic level of hydrogen peroxide induced premature senescence in NIH 3T3 cells and increased endogenous caveolin-1 expression (33). Quercetin and vitamin E, two antioxidant agents, successfully prevented the premature senescent phenotype and the up-regulation of caveolin-1 induced by hydrogen peroxide (33). Interestingly, premature senescence induced by hydrogen peroxide was greatly reduced in NIH 3T3 cells when the up-regulation of caveolin-1 expression was prevented by antisense caveolin-1 mRNA (33). Induction of premature senescence was recovered when caveolin-1 levels were restored. Taken together, these results clearly indicate a central role for caveolin-1 in the signaling events that regulate oxidative stress–induced premature senescence.

However, the signaling machinery that links oxidative stress to caveolin-1-mediated premature senescence remains unknown. Here, we show that the following signaling pathway regulates the

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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oxidant-induced activation of the caveolin-1 gene: subcytotoxic oxidative stress → activation of p38 mitogen-activated protein kinase (MAPK) → Sp1-mediated activation of GC-rich caveolin-1 promoter elements → caveolin-1 gene transcription → premature senescence.

Materials and Methods

Materials. Antibodies and their sources were as follows: anti-caveolin-1 immunoglobulin G (IgG; mouse monoclonal antibody 2297) was from Becton Dickinson Biosciences (San Jose, CA); anti–p38 MAPK and anti–phosphospecific p38 MAPK (polyclonal antibodies) were from New England Biolabs, Inc. (Ipswich, MA); anti-p21 (polyclonal antibody) and anti-Sp1 (polyclonal antibody PEP2) for immunoblotting analysis and anti-Sp1 (polyclonal antibody PEP2-X) for chromatin immunoprecipitation assays were from Santa Cruz Biotechnology (Santa Cruz, CA). The p38 MAPK inhibitor SB203580 and SB202474 (an inactive control compound) were from Calbiochem, Inc. (San Diego, CA). All other biochemicals used were of the highest purity available and were obtained from regular commercial sources.

Cell culture. NIH 3T3 cells were grown in DMEM supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% donor bovine calf serum. MCF-7 cells (from American Type Culture Collection, Manassas, VA) were grown in DMEM supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% FCS. Human mammary epithelial cells (from Cambrex, Walkersville, MD) were grown in Mammary Epithelial Basal Medium supplemented with glutamine, bovine pituitary extracts (70 μg/mL), epidermal growth factor (20 ng/mL), hydrocortisone (5 μg/mL), insulin (10 μg/mL), gentamicin (50 μg/mL), and amphotericin (2.5 μg/mL).

Generation of caveolin-1 promoter reporter constructs. Caveolin-1 promoter deletion mutants were generated by PCR with the following forward primers: Cav-1 (−1,296/−1), ggccgg**ggtacc**cccaggttactggtcttttct; Cav-1 (−800/−1), ggccgg**ggtacc**tcgtcgatcttgagccaaga; Cav-1 (−372/−1), ggccgg**ggtacc**aacacgcgctctcccctggat; Cav-1 (−222/−1), ggccgg**ggtacc**caagaaccttggggatgtgcc; Cav-1 (−150/−1), ggccgg**ggtacc**cctggcgaacagccaaagaggc; and Cav-1 (−91/−1), ggccgg**ggtacc**cctctatacaatacaagatct. The following reverse primer was used for the generation of all deletion mutant constructs: ggccgg*ccatgg*gctggcgcgtggctggctgca. *Kpn*I restriction sites are in boldface; the *Nco*I restriction site is in italic. Wild-type and mutated Cav-1 (−244/−222) and Cav-1 (−124/−101) luciferase constructs were generated by PCR with the forward primers illustrated in Table 1 containing *Kpn*I restriction sites. The following reverse primer containing an *Nco*I restriction site (shown in italic) was used to generate both Cav-1 (−244/−222) and Cav-1 (−124/−101) promoter constructs: ggccgg*ccatgg*tggctttaccaacaggtaccgg. All PCR products were cloned with *Kpn*I/*Nco*I into the luciferase-based vector pTA-luc (Clontech, Mountain View, CA).

Table 1. Oligonucleotide sequences from the mouse caveolin-1 promoter for generation of heterologous reporter constructs and electrophoretic mobility shift assay

Oligonucleotide	Sequence (5′ → 3′)
	C G
Cav-1 (−244/−222)	GGC ACT *CCC CGC CC*T CTG CTG CC
Cav-1 (−244/−222) MUT	GGC ACT **CTT TTT TT**T CTG CTG CC
	T
Cav-1 (−124/−101)	CAG CCA *CCG CCC CCC GCC* AGC GC
Cav-1 (−124/−101) MUT	CAG CCA **TTT TTT TT**C GCC AGC GC

NOTE: GC-boxes are shown in italic; introduced mutations are shown in boldface; underlying nucleotides indicate nonconserved residues between the mouse and human caveolin-1 promoters (the residue found in the human caveolin-1 promoter is shown above the mouse sequence).

Luciferase reporter assay. Cells were seeded in 60-mm dishes at 270,000 per dish. The following day, cells were transiently transfected, using a modified calcium-phosphate precipitation method, with 2 μg of the caveolin-1 promoter luciferase reporter constructs or the luciferase reporter plasmid pTA-luc and 1 μg of a β-galactosidase-expressing construct. Twenty-four hours posttransfection, cells were rinsed twice with PBS and incubated in medium containing 150 μmol/L H_2O_2, with or without 300 μmol/L quercetin, for 2 hours. Cells were then washed twice and incubated in complete medium at 37°C for an additional 48 hours. Cells were then lysed in 500 μL of extraction buffer; 200 μL were used to measure luciferase activity and 150 μL were used to measure β-galactosidase activity as previously described (36). Three independent experiments were done for each condition.

Electrophoretic mobility shift assays. Nuclear protein extracts (5 μg) from untreated and H_2O_2-treated (150 μmol/L for 2 hours) NIH 3T3 cells were incubated with the following 3′-end biotin-labeled double-stranded oligonucleotides containing a GC-rich box (in boldface): Cav-1 (−244/−222), ggcact**ccccgccc**tctgctgcc; Cav-1 (−124/−101), cagcca**ccgccccccgc**cagcgc.

The incubation was done for 15 minutes at room temperature in the following buffer: 100 mmol/L Tris-HCl (pH 8.0), 50% glycerol, 10 mmol/L EDTA, 10 mmol/L DTT, and 1 mg/mL poly(deoxyinosinic-deoxycytidylic acid). To resolve protein-DNA complexes, the reaction mix was run on a 5% nondenaturing gel in 1× Tris-borate EDTA. The gel was transferred to the positively charged Biodyne B nylon membrane (Pierce Biotechnology, Inc., Rockford, IL) and the membrane was developed using the LightShift Chemiluminescent EMSA Kit from Pierce Biotechnology according to the instructions of the manufacturer. For competition assays, nuclear extracts were incubated with a 100-fold molar excess of unlabeled double-stranded competitor oligonucleotides. A representative blot is shown from three independent experiments.

Chromatin immunoprecipitation assays. NIH 3T3 cells were left untreated or treated with H_2O_2 (150 μmol/L) for 2 hours. Chromatin immunoprecipitation assay was done 48 hours after oxidative stress with the chromatin immunoprecipitation assay kit from Upstate Cell Signaling Solutions (Temecula, CA). Briefly, cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C. Cells were washed with cold PBS, harvested, and the DNA fragmented into ~250- to 1,000-bp fragments by sonication using the Cole Parmer Ultrasonic Processor sonicator (model CPX 750). Chromatin immunoprecipitation lysates were diluted and precleared with salmon sperm DNA-protein A-agarose beads for 30 minutes at 4°C. One tenth of the supernatant was taken as input DNA for PCR reactions to show equal chromatin content before immunoprecipitation. The rest of the supernatant was then incubated overnight at 4°C with anti-Sp1 IgGs and salmon sperm DNA-protein A-agarose beads. Following washes, the bound DNA was eluted from the immune complex and purified using the Qiagen (Valencia, CA) PCR purification kit according to the instructions of the manufacturer. PCR was done in the exponential linear zone of amplification with the following primers, which flank the two GC-rich boxes in the caveolin-1 gene promoter: $^{-264}$caggctctcagctccccgccg^{-244} (forward) and $^{-84}$gtatagagggggggaaaggcgc^{-104} (reverse). A representative gel is shown from two independent experiments.

Immunoblotting. Cells were collected in boiling sample buffer. Cellular proteins were resolved by SDS-PAGE (12.5% acrylamide) and transferred to BA83 nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Blots were incubated for 2 hours in TBST [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.2% Tween 20] containing 2% powdered skim milk and 1% bovine serum albumin. After three washes with TBST, membranes were incubated for 2 hours with the primary antibody and for 1 hour with horseradish peroxidase–conjugated goat anti-rabbit/mouse IgG. Bound antibodies were detected with an enhanced chemiluminescence detection kit (Pierce).

Acid β-galactosidase staining. Cells were subjected to acid β-galactosidase staining using the Senescence β-galactosidase Staining Kit

(Cell Signaling, Danvers, MA) according to the recommendations of the manufacturer. Briefly, cells were washed twice with PBS and fixed with the fixative solution for 15 minutes. Then, cells were washed twice with PBS and incubated overnight at 37°C with the staining solution. Cells were then examined for the development of blue color. Cells were photographed at low magnification (×10) with a BX50WI Olympus Optical light microscope (Tokyo, Japan).

Adenovirus infection. Ad-cav-1 and Ad-tTA were as previously described (37). Subconfluent MCF-7 cells were coinfected with Ad-cav-1 (500 plaque-forming units/cell) together with Ad-tTA (transactivator; 100 plaque-forming units/cell) for 1 hour in serum-free DMEM and grown in complete medium for the indicated period of time. Cells were then lysed and subjected to immunoblotting analysis with anti-caveolin-1 IgG or 4′, 6-diamidino-2-phenylindole (DAPI) staining or terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis.

DAPI staining. Cells were washed twice with PBS and fixed with 3.7% paraformaldehyde at room temperature for 20 minutes. Then, cells were incubated with RNase A (10 μg/mL in PBS) for 10 minutes and with DAPI (1 μg/mL in PBS) for 10 minutes. Nuclear morphology was examined with an Olympus Provis fluorescent microscope. Apoptotic nuclei were counted from at least 10 randomly chosen fields.

TUNEL analysis. After fixation, cell death was detected by the TUNEL assay using the TUNEL Apoptosis Detection Kit (Upstate Cell Signaling Solutions) according to the instructions of the manufacturer. Labeling indices were obtained by counting TUNEL-positive cells from at least 10 randomly chosen fields and expressed as relative percentage or positive cell number per field. Costaining with propidium iodide was done as a control. Fixed cells treated with DNase I were used as a positive control for TUNEL staining.

Results

The mouse caveolin-1 promoter sequences −372/−222 and −150/−91 possess oxidative stress responsive elements. We have previously shown that subcytotoxic levels of hydrogen peroxide (150 μmol/L for 2 hours) stimulated caveolin-1 protein expression within 48 to 72 hours in NIH 3T3 cells and that such up-regulation was required for the induction of premature senescence (33). To investigate whether up-regulation of caveolin-1 protein expression occurs through activation of the caveolin-1 promoter, functional 5′ deletion analysis of the caveolin-1 promoter was done by generating a series of deletion mutants fused to the luciferase gene (Supplementary Fig. S1*A*). These constructs were transiently transfected in NIH 3T3 cells treated with or without 150 μmol/L hydrogen peroxide for 2 hours. Figure 1*A* shows that Cav-1 (−1,296/−1), Cav-1 (−800/−1), and Cav-1 (−372/−1) were equally activated by oxidative stress (~15-fold induction). In contrast, Cav-1 (−222/−1) and Cav-1 (−150/−1) showed only a 4-fold induction upon hydrogen peroxide treatment (Fig. 1*A*). Finally, the first 91 nucleotides of the caveolin-1 promoter did not respond

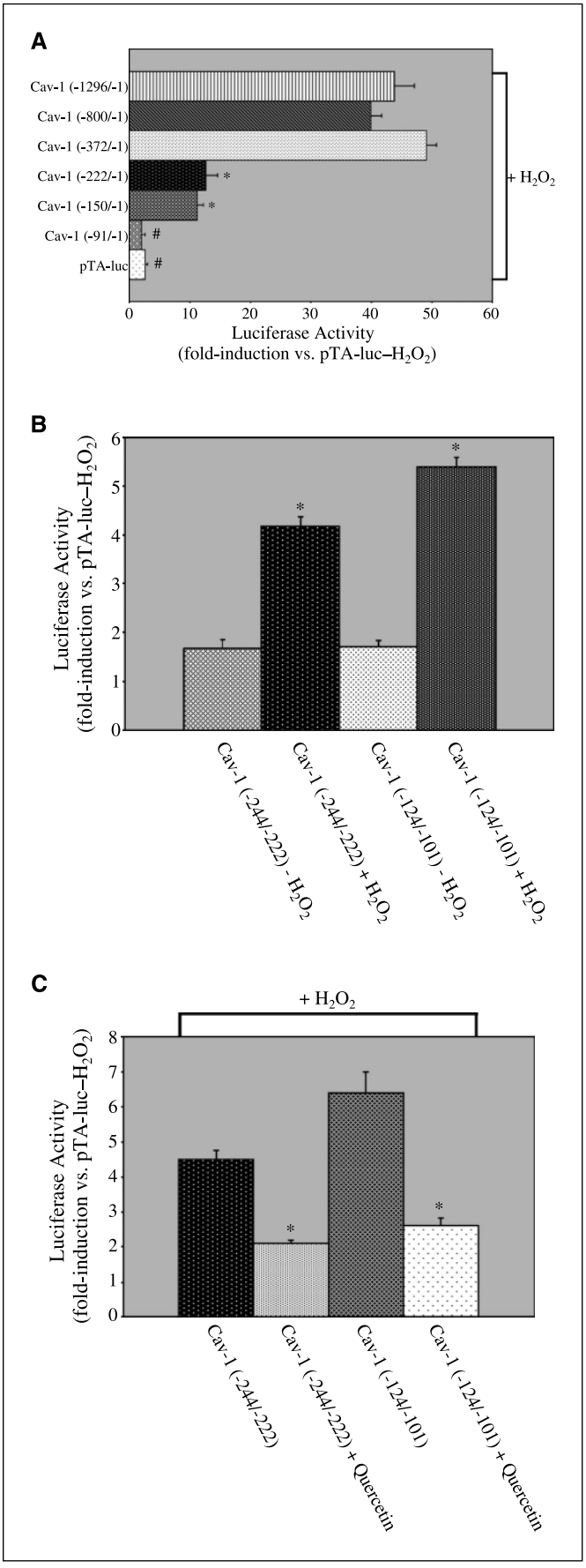
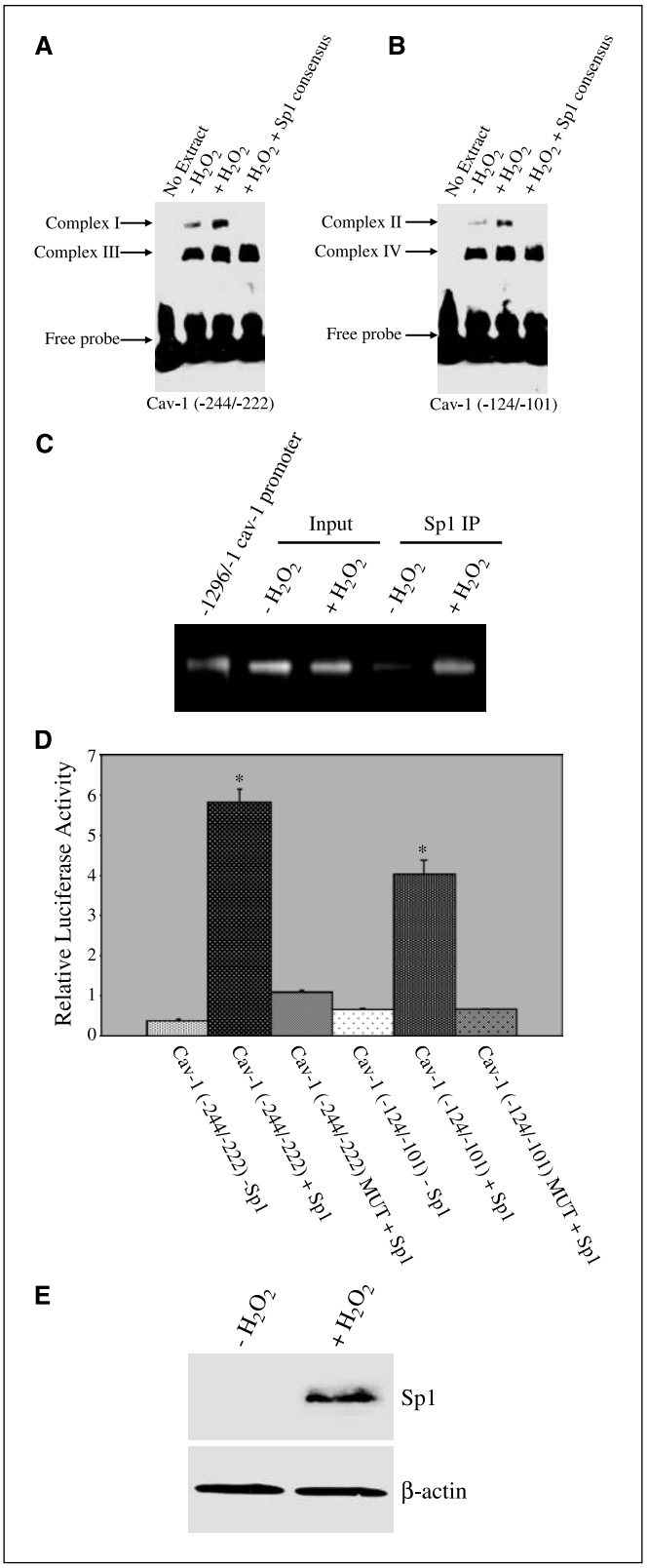


Figure 1. Oxidative stress activates the caveolin-1 promoter by acting through two GC-rich boxes. *A,* luciferase assay. Caveolin-1 promoter deletion mutant constructs were transiently transfected in NIH 3T3 cells. pTA-luc alone was used as a control. Twenty-four hours after transfection, cells were treated with or without 150 μmol/L H_2O_2 for 2 hours. Cells were collected 48 hours after oxidative stress and luciferase activity was measured. *Columns,* mean; *bars,* SE. *, #, $P < 0.001$. *B* and *C,* luciferase assay. The caveolin-1 promoter −244/−222 and −124/−101 regions, both containing a GC-rich box, were cloned upstream of the luciferase gene in the pTA-luc vector. These constructs were transiently transfected in NIH 3T3 cells. pTA-luc alone was used as a control. Twenty-four hours after transfection, cells were treated with or without 150 μmol/L H_2O_2 for 2 hours (*B*). *C,* cells were treated with hydrogen peroxide as in (*B*) in the presence or absence of 300 μmol/L quercetin. Cells were collected 48 hours after oxidative stress and luciferase activity was measured. *Columns,* mean; *bars,* SE. *, $P < 0.001$.

to H_2O_2 (Fig. 1*A*). Thus, these results indicate that the mouse caveolin-1 promoter has two oxidative stress responsive elements: one being located between nucleotides −372 and −222 and one between nucleotides −150 and −91.



Two GC-rich elements within the caveolin-1 promoter respond to oxidative stress. Analysis of the mouse caveolin-1 promoter sequence revealed that two GC-rich boxes are located within the two oxidative stress responsive regions identified in Fig. 1*A* (Supplementary Fig. S1*B*). To determine whether these two GC-rich boxes were indeed oxidant-responsive elements, sequences containing the GC-rich boxes (−244/−222 and −124/−101; see Table 1) were fused to the luciferase gene, and their ability to respond to hydrogen peroxide was tested after expression in NIH 3T3 cells. Figure 1*B* illustrates that the two sequences (−244/−222 and −124/−101) of the caveolin-1 promoter contain the information necessary to respond to oxidative stress. In support to this finding, their ability to be activated by hydrogen peroxide was significantly inhibited when the cells were treated with hydrogen peroxide in the presence of the antioxidant quercetin (Fig. 1*C*).

Oxidative stress stimulates binding of Sp1 to GC-rich elements within the caveolin-1 promoter. Transcription factors belonging to the Sp family are known to bind to GC-rich boxes. The family member Sp1 has been shown to mediate oxidative stress–induced gene transcription (38–40). Thus, we decided to look at whether Sp1 may represent the transcription factor that activates the caveolin-1 gene after oxidative stress. We first show in electrophoretic mobility shift studies that the −244/−222 and −124/−101 caveolin-1 promoter sequences formed nucleoprotein complexes (complex I and complex II, respectively) that were significantly up-regulated 48 hours after treatment with hydrogen peroxide (150 μmol/L) for 2 hours (Fig. 2*A* and *B*). Incubation with excess unlabeled double-stranded oligonucleotides representing the Sp1 consensus sequence totally prevented the formation of both complex I and complex II (Fig. 2*A* and *B*). We also observed a second complex with both caveolin-1 promoter sequences (−244/−222 and −124/−101) that was not influenced

Figure 2. Oxidative stress stimulates the binding of Sp1 to GC-rich boxes of the caveolin-1 promoter. *A* and *B,* electrophoretic mobility shift studies. Electrophoretic mobility shift assays were done with nuclear extracts from untreated and H_2O_2-treated (150 μmol/L for 2 hours) NIH 3T3 cells 48 hours after oxidative stress. Nuclear extracts were incubated with either Cav-1 (−244/−222; *A*) or Cav-1 (−124/−101; *B*) biotin-labeled oligonucleotides. Lack of nuclear extract was used as a negative control. Note that two nucleoprotein complexes were identified in (*A*) (complex I and complex III) and two in (*B*) (complex II and complex IV). Incubation with excess unlabeled Sp1 consensus oligonucleotides was done to show specificity of complexes I and II. *C,* chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay was done on chromatin derived from untreated or hydrogen peroxide–treated (150 μmol/L for 2 hours) NIH 3T3 cells 48 hours after oxidative stress using an antibody probe specific for Sp1. PCR was done using primers surrounding the region of the caveolin-1 promoter containing the two GC-rich boxes (see Materials and Methods for details). Amplification of input DNA from both untreated and H_2O_2-treated cells was done before immunoprecipitation. A vector containing the entire caveolin-1 promoter sequence was used as a positive control for PCR. *D,* luciferase assay. The caveolin-1 promoter −244/−222 and −124/−101 regions, both containing a GC-rich box, were cloned upstream of the luciferase gene in the pTA-luc vector. The same caveolin-1 promoter regions in which guanines and cytosines within the GC-rich box were substituted with thymines (see Table 1) were cloned upstream of the luciferase gene [*Cav-1 (−244/−222) MUT* and *Cav-1 (−124/−101) MUT*]. These constructs were transiently cotransfected in NIH 3T3 cells together with a Sp1-expressing vector (+ *Sp1*). Cotransfection of the luciferase constructs with the vector used to drive Sp1 expression was done as a control (− *Sp1*). Cells were collected 48 hours after transfection and luciferase activity was measured. *Columns,* mean; *bars,* SE. *, $P < 0.001$. *E,* immunoblotting. NIH 3T3 cells were treated with or without hydrogen peroxide (150 μmol/L) for 2 hours. After 48 hours, cells were collected and the expression of endogenous Sp1 was evaluated by immunoblotting analysis with an antibody probe specific for Sp1. Immunoblotting with anti-β-actin IgGs was done to show equal loading.

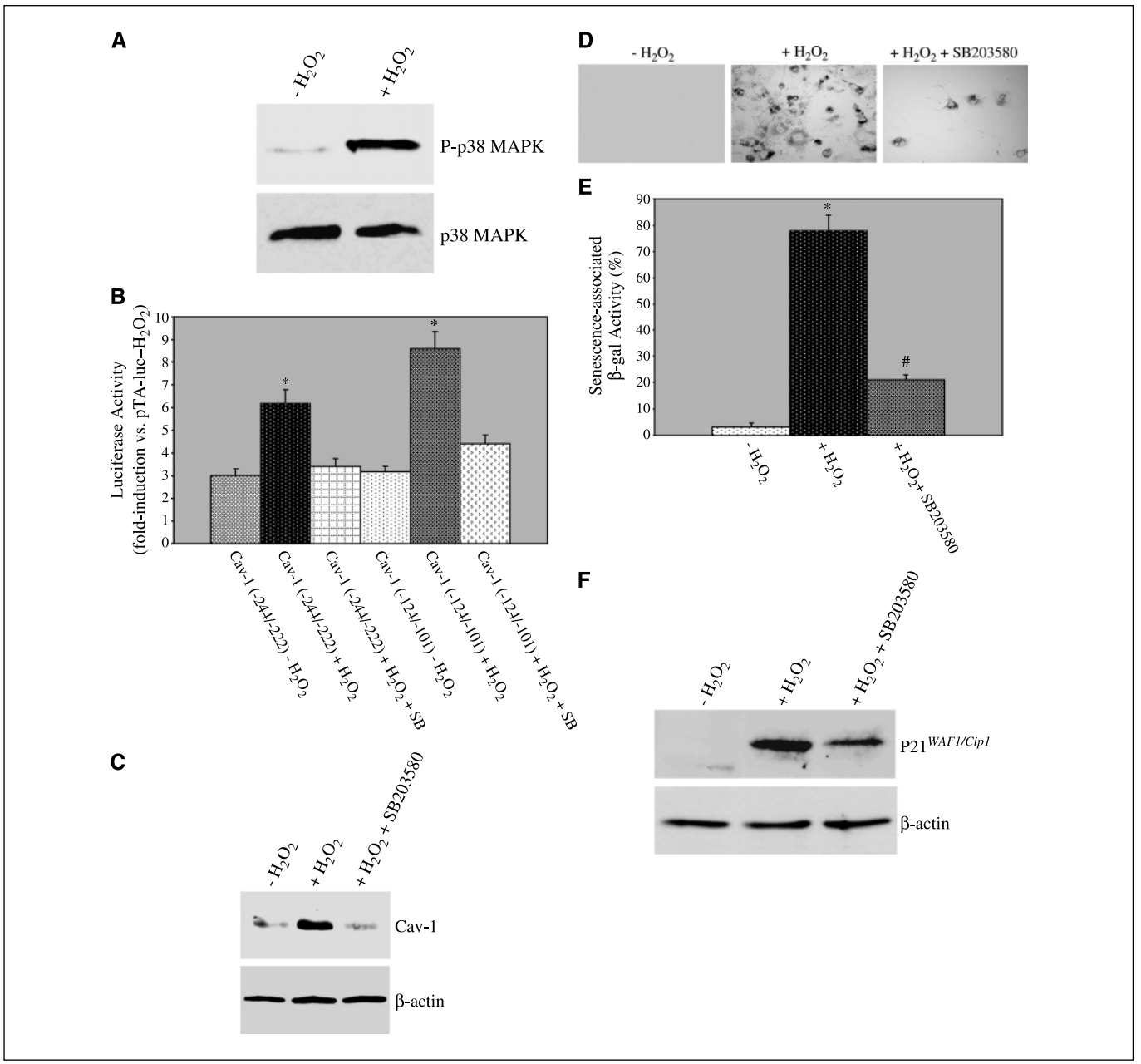


Figure 3. Inhibition of the p38 MAPK pathway prevents the oxidant-induced activation of GC-rich boxes within the caveolin-1 promoter, up-regulation of caveolin-1 protein expression, and development of premature senescence. *A,* immunoblotting. NIH 3T3 cells were treated with or without hydrogen peroxide (150 μmol/L) for 2 hours. After 48 hours, cells were collected and the expression of activated p38 MAPK was evaluated by immunoblotting analysis with an antibody probe specific for the phosphorylated form of p38 MAPK (*top*). *Bottom,* immunoblotting with an antibody probe that recognizes total p38 MAPK expression. *B,* luciferase assay. The caveolin-1 promoter −244/−222 and −124/−101 luciferase constructs were transiently transfected in NIH 3T3 cells. Twenty-four hours after transfection, cells were treated with 150 μmol/L H_2O_2 for 2 hours in the presence or absence of the p38 MAPK inhibitor SB203580. Cells were then cultured for 48 hours with or without SB203580 and luciferase activity was measured. Untreated cells were used as a control. *Columns,* mean; *bars,* SE. *, $P < 0.001$. *C,* immunoblotting. NIH 3T3 cells were treated with hydrogen peroxide (150 μmol/L) for 2 hours in the presence or absence of the SB203580 inhibitor. Cells were cultured for 48 hours with or without SB203580. Cells were then collected and the expression of endogenous caveolin-1 was evaluated by immunoblotting analysis with anti-caveolin-1 IgGs. Untreated cells were used as a control. Immunoblotting with anti-β-actin IgGs was done to show equal loading. *D*, acid β-galactosidase activity assay. NIH 3T3 cells were treated with 150 μmol/L hydrogen peroxide for 2 hours in the presence or absence of the p38 MAPK inhibitor SB203580 and recovered for 7 days with or without SB203580. Untreated cells were used as a control. Cells were then subjected to senescence-associated β-galactosidase activity assay and observed under a BX50WI Olympus Optical light microscope at a magnification of ×10. Representative field. *E*, quantitation of the acid β-galactosidase activity assay shown in (*D*). *Columns*, mean; bars, SE. *, #, $P < 0.001$. *F*, immunoblotting. Cells were treated as in (*D*). Cells were then collected and the expression of endogenous $p21^{WAF1/Cip1}$ was evaluated by immunoblotting analysis with an antibody probe specific for $p21^{WAF1/Cip1}$. Untreated cells were used as a control. Immunoblotting with an antibody probe specific for β-actin was done to show equal loading.

by hydrogen peroxide and the unlabeled oligonucleotide representing the Sp1 consensus sequence, and therefore must be regarded as nonspecific (complex III and complex IV in Fig. 2*A* and *B*, respectively).

To directly show binding of Sp1 to the GC-rich boxes *in vivo*, a chromatin immunoprecipitation analysis was done on chromatin from untreated and H_2O_2-treated cells using an antibody probe specific for Sp1. Figure 2*C* shows increased binding of Sp1 to the

caveolin-1 promoter region containing the two GC-rich boxes 48 hours after treatment with hydrogen peroxide.

Sp1 potently transactivates the Cav-1 (−244/−222) and Cav-1 (−124/−101) GC-rich elements. After the identification of Sp1 binding to GC-rich boxes of the caveolin-1 promoter, we next examined the functional effect of Sp1 on these two elements in transient transfections. We show in Fig. 2*D* that overexpression of Sp1 potentially stimulated both Cav-1 (−244/−222) and Cav-1 (−124/−101) elements, showing that these sequences are highly reactive to Sp1. In support to these findings, Sp1 failed to activate luciferase constructs carrying the two GC-rich boxes in which guanines and cytosines were substituted with thymines (Fig. 2*D*). In addition, we show in Fig. 2*E* that Sp1 protein expression is dramatically up-regulated 48 hours after treatment with hydrogen peroxide (150 μmol/L for 2 hours) in NIH 3T3 cells. Taken together, these data indicate that Sp1 mediates the activation of the caveolin-1 gene after oxidative stress in fibroblasts.

p38 MAPK promotes the oxidant-induced Sp1-mediated activation of the caveolin-1 gene promoter. To gain mechanistic insights into the oxidant-mediated activation of the caveolin-1 gene, we investigated the potential role of p38 MAPK. We decided to focus on p38 MAPK because this kinase is a well-known mediator of cellular stress in a variety of cell types. We show in Fig. 3*A* that p38 MAPK is dramatically activated 48 hours after treatment with subcytotoxic levels of hydrogen peroxide (150 μmol/L) for 2 hours. Inhibition of p38 MAPK signaling with the specific SB203580 inhibitor totally prevented the H_2O_2-mediated activation of the two Sp1 binding sites of the caveolin-1 promoter (−244/−222 and −124/−101; Fig. 3*B*). SB203580 prevented also the activation of the entire caveolin-1 promoter (Cav-1 −1,296/−1) by hydrogen peroxide (data not shown). SB202474, an inactive control compound, had no effect on GC-rich boxes of the caveolin-1 gene (data not shown). In addition, treatment with PD98059, a specific p42/44 MAPK pathway inhibitor, failed to inhibit the hydrogen peroxide–initiated activation of the GC-rich boxes of the caveolin-1 promoter (data not shown).

Inhibition of p38 MAPK prevents oxidative stress–induced up-regulation of caveolin-1 protein expression, development of premature senescence, and up-regulation of p21$^{WAF1/Cip1}$. In Fig. 3*B*, we show that p38 MAPK mediated the hydrogen peroxide–induced up-regulation of the caveolin-1 promoter. To support this data, we looked at the effect that the SB203580 p38 MAPK inhibitor has on caveolin-1 protein expression after oxidative stress. NIH 3T3 cells were treated with subcytotoxic levels of hydrogen peroxide (150 μmol/L) for 2 hours in the presence or absence of SB203580. Untreated cells were used as a control. Figure 3*C* illustrates that, 48 hours after oxidative stress, the H_2O_2-mediated up-regulation of caveolin-1 expression was totally prevented by the p38 MAPK inhibitor.

We next examined the functional consequence of preventing the oxidant-induced p38 MAPK/Sp1–mediated up-regulation of caveolin-1 expression. Subcytotoxic levels of hydrogen peroxide are known to induce premature senescence in culture. We have previously shown that treatment with 150 μmol/L H_2O_2 for 2 hours is sufficient to promote premature senescence in NIH 3T3 cells 7 to 10 days after oxidative stress (33). It is also well known that senescent cells display a typical large and flat morphology (41–43). Thus, we evaluated the cell morphology of NIH 3T3 cells treated with subcytotoxic levels of hydrogen peroxide in the presence or absence of p38 MAPK inhibitor 7 days after oxidative stress. Representative cells are shown in Supplementary Fig. S2*A*. Light

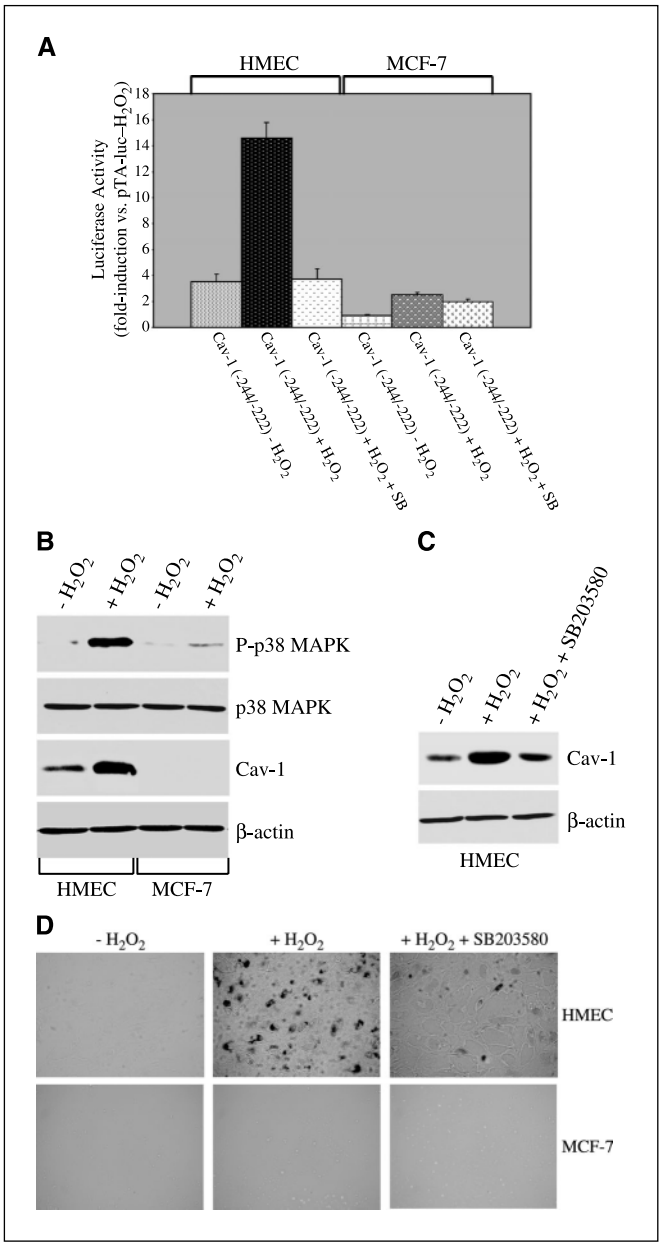


Figure 4. Oxidative stress activates the caveolin-1 promoter, caveolin-1 protein expression, and p38 MAPK, and induces premature senescence in human mammary epithelial cells but not in MCF-7 cells. *A,* luciferase assay. The caveolin-1 promoter −244/−222 luciferase construct was transiently transfected in human mammary epithelial cells (*HMEC*) and MCF-7 cells. Twenty-four hours after transfection, cells were treated with 450 μmol/L H_2O_2 for 2 hours in the presence or absence of the p38 MAPK inhibitor SB203580. Cells were then cultured for 24 hours with or without SB203580 and luciferase activity was measured. Untreated cells were used as a control. *Columns,* mean; *bars,* SE. *, $P < 0.001$. *B* and *C,* immunoblotting. Human mammary epithelial cells (*B* and *C*) and MCF-7 (*B*) cells were treated with or without hydrogen peroxide (450 μmol/L) for 2 hours in the presence or absence of the p38 MAPK inhibitor SB203580. Cells were recovered for 24 hours in the presence or absence of SB203580 and collected. Expression of activated p38 MAPK and caveolin-1 was evaluated by immunoblotting analysis with antibody probes specific for the phosphorylated form of p38 MAPK and caveolin-1. Immunoblotting with antibody probes that recognize total p38 MAPK expression and β-actin was done as a control. *D,* acid β-galactosidase activity assay. Human mammary epithelial cells and MCF-7 cells were treated with 450 μmol/L hydrogen peroxide for 2 hours in the presence or absence of the p38 MAPK inhibitor SB203580 and recovered for 7 days with or without SB203580. Untreated cells were used as a control. Cells were then subjected to senescence-associated β-galactosidase activity assay and observed under a BX50WI Olympus Optical light microscope at a magnification of ×10. Representative field.

microscopy analysis indicated that 89 ± 6% of NIH 3T3 cells treated with hydrogen peroxide without SB203580 showed a large and flat morphology, as compared with 26 ± 3% of cells treated with H_2O_2 in the presence of p38 MAPK inhibitor (Supplementary Fig. S2*B*). Caveolin-1 expression remained elevated 7 days after oxidative stress and SB203580 prevented the H_2O_2-induced up-regulation of caveolin-1 7 days after oxidative stress (data not shown).

We next examined whether inhibition of the p38 MAPK signaling prevented the expression of acid β-galactosidase enzymatic activity. Acid β-galactosidase activity (at pH 6) is a well-established biochemical marker that is associated with the senescent cell phenotype (41–43). A representative field is shown in Fig. 3*D*. Quantitation of this staining indicates that 78 ± 6% of NIH 3T3 cells treated with H_2O_2 in the absence of SB203580 were positive for acid β-galactosidase enzymatic activity, as compared with 21 ± 2% of cells treated with hydrogen peroxide in the presence of SB203580 (Fig. 3*E*).

The p53/p21 pathway is activated in senescent cells. In addition, we have previously shown that overexpression of caveolin-1 is sufficient to activate p53 and up-regulate $p21^{WAF1/Cip1}$ expression (34). To further prove that inhibition of the p38 MAPK/Sp1–mediated up-regulation of caveolin-1 expression prevents the development of premature senescence, we evaluated the effect of inhibition of p38 MAPK on the cellular levels of $p21^{WAF1/Cip1}$. Figure 3*F* shows that $p21^{WAF1/Cip1}$ expression was up-regulated by oxidative stress and that this effect was dramatically prevented by treatment with the SB203580 inhibitor.

Oxidants promote p38 MAPK–mediated up-regulation of caveolin-1 and premature senescence in normal mammary epithelial cells. To evaluate whether the p38 MAPK–mediated activation of the caveolin-1 promoter and up-regulation of caveolin-1 protein expression after oxidative stress were cell type specific, we moved our focus to normal human mammary epithelial cells. We show that oxidative stress dramatically activated the Sp1 consensus site −244/−222 of the caveolin-1 promoter (Fig. 4*A*) and up-regulated caveolin-1 protein expression (Fig. 4*B*) in human mammary epithelial cells. This effect was p38 MAPK dependent, as shown by lack of activation of the Sp1 consensus site and up-regulation of caveolin-1 protein expression after oxidative stress in the presence of the SB203580 p38 MAPK inhibitor (Fig. 4*A* and *C*, respectively). Consistent with these data, oxidative stress activated p38 MAPK in human mammary epithelial cells (Fig. 4*B*). Finally, we show in Fig. 4*D* that oxidative stress induced premature senescence in human mammary epithelial cells and that this effect was p38 MAPK dependent (quantification of this staining is shown in Supplementary Fig. S3*A*).

The oxidant-induced p38 MAPK-caveolin-1 pathway is repressed in MCF-7 cells, which undergo apoptosis rather than premature senescence after oxidative stress. Because MCF-7 breast cancer cells are known to lack endogenous caveolin-1 expression, we reasoned that these cells may differentially respond to oxidative stress as compared with their normal counterparts (human mammary epithelial cells). We show in Fig. 4*A* that oxidative stress failed to activate the Sp1 consensus site −244/−222 of the caveolin-1 promoter. Consistent with this data, treatment with hydrogen peroxide did not up-regulate caveolin-1 protein expression, nor did it activate p38 MAPK (Fig. 4*B*). In addition, oxidative stress failed to induce premature senescence in MCF-7 cells (Fig. 4*D*). In contrast to human mammary epithelial cells, MCF-7 cells died by apoptosis after treatment with hydrogen peroxide, as shown by reduced cell number (Fig. 5*A*), analysis of cell morphology (Supplementary Fig. S3*B*), nuclear condensation

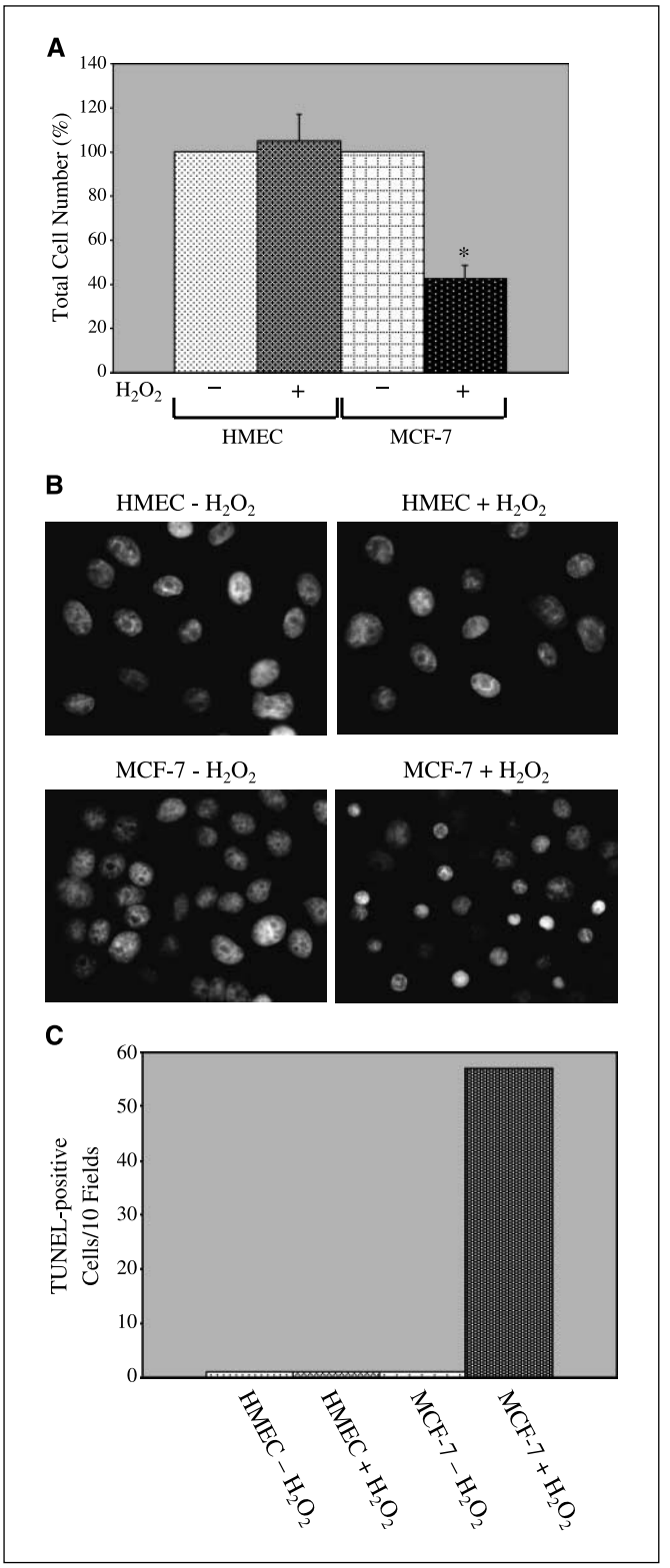
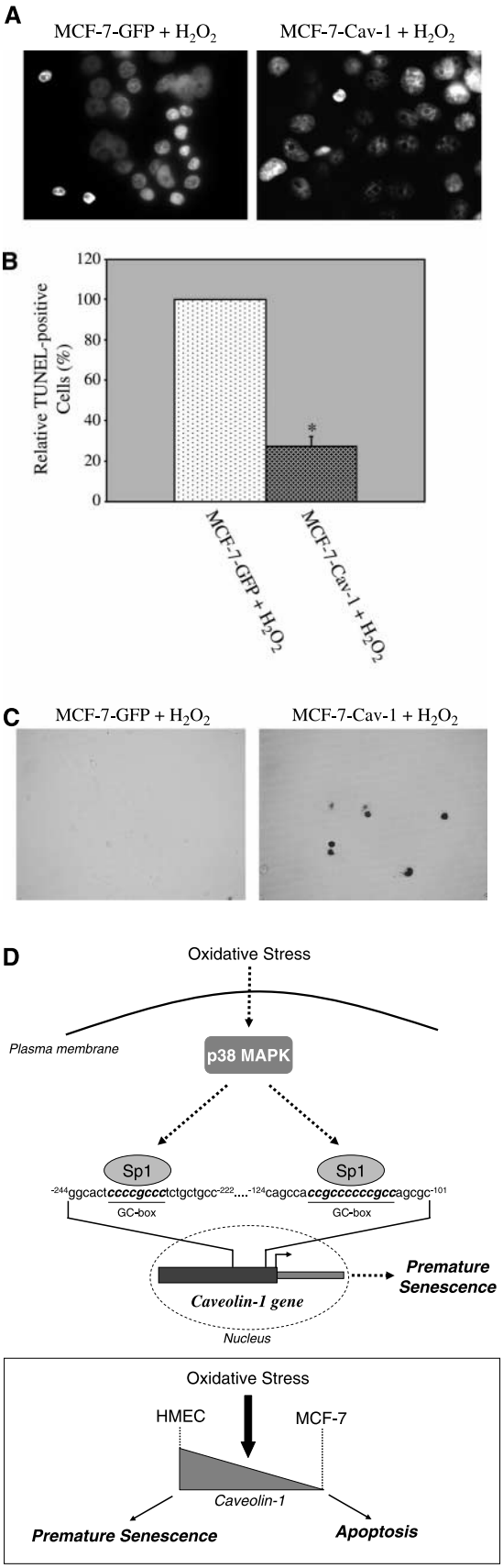


Figure 5. Oxidative stress induces apoptosis in MCF-7 cells. *A,* human mammary epithelial cells and MCF-7 cells were treated with or without hydrogen peroxide (450 μmol/L) for 2 hours and recovered for 24 hours. Cells were counted from 10 independent fields and total cell number was expressed as percentage. *Columns,* mean; *bars,* SE. *, $P < 0.005$. *B,* DAPI staining. Cells were treated as in (*A*). Nuclear morphology was examined by DAPI staining. Representative field. *C,* TUNEL analysis. Cells were treated as in (*A*). Apoptotic cells were detected by TUNEL analysis. Total number of TUNEL-positive cells per 10 fields.



(Fig. 5*B*), and TUNEL staining (Fig. 5*C*). Loss of caveolin-1 expression seems to play a key role in oxidant-induced apoptosis in MCF-7 cells, as shown by the significant prevention of apoptosis in MCF-7 cells reexpressing caveolin-1 after treatment with hydrogen peroxide (Supplementary Fig. S4*A* and *B*; Fig. 6*A* and *B*). Interestingly, reexpression of caveolin-1 in MCF-7 cells was sufficient to induce premature senescence after oxidative stress (Fig. 6*C*).

Discussion

Most cells cannot divide indefinitely due to a process termed cellular senescence. Cellular senescence seems to be a fundamental feature of somatic cells, with the exception of most tumor cells and certain stem cells. Senescent cells undergo irreversible growth arrest, become enlarged, and display a number of molecular changes. Oxidative stress has been shown to induce premature senescence in culture (43–45). Because a number of molecular changes observed in senescent cells occur in somatic cells during the aging process, investigating the molecular mechanisms underlying oxidative stress induced premature senescence will allow us to better understand the more complicated aging process.

Caveolin-1 expression seems to play a major role in stress-induced premature senescence. In fact, we have previously shown that up-regulation of caveolin-1 expression was required for oxidative stress–induced premature senescence in mouse fibroblasts (33). However, the signaling machinery that links oxidative stress to up-regulation of caveolin-1 expression remains to be determined. Here, we show that oxidants activated caveolin-1 gene transcription through a Sp1-mediated mechanism. More specifically, oxidative stress stimulated binding of Sp1 to two GC-rich boxes within the caveolin-1 promoter. This effect may not be limited to the mouse caveolin-1 promoter. In fact, the Cav-1 (−244/−222) GC-rich box is 100% conserved and the Cav-1 (−124/−101) GC-rich box is 92% conserved between mouse and human (Table 1).

The maintenance of a "physiologic redox tone" is essential to prevent the degenerative processes associated with aging. Dietary antioxidants are believed to prevent and/or contain oxidative damages induced by oxidative stress (46, 47). We show here that quercetin, a flavanoid found in foods of plant origin, prevented the oxidative stress–induced activation of the two GC-rich boxes contained within the Cav-1 (−244/−222) and Cav-1 (−124/−101)

Figure 6. Reexpression of caveolin-1 in MCF-7 cells prevents oxidant-induced apoptosis and promotes premature senescence. *A,* DAPI staining. MCF-7 cells were infected with green fluorescent protein (*GFP*) or caveolin-1 using adenovirus. Cells were treated with 450 μmol/L hydrogen peroxide for 2 hours and recovered for 24 hours. Nuclear morphology was examined by DAPI staining. Representative field. *B,* TUNEL analysis. Cells were treated as in (*A*). Apoptotic cells were detected by TUNEL analysis. Total number of TUNEL-positive cells is expressed as relative percentage. *Columns,* mean; *bars,* SE. *, $P < 0.005$. *C,* acid β-galactosidase activity assay. Cells were treated as in (*A*) and recovered for 7 days. Cells were then subjected to senescence-associated β-galactosidase activity assay and observed under a BX50WI Olympus Optical light microscope at a magnification of ×10. Representative field. *D,* schematic diagram summarizing the oxidant-induced up-regulation of caveolin-1 expression. Oxidative stress activates p38 MAPK. Activation of the p38 MAPK pathway promotes caveolin-1 gene transcription through binding of Sp1 to GC-rich boxes within the caveolin-1 promoter. Oxidant-induced p38 MAPK/Sp1–mediated up-regulation of caveolin-1 expression is required for the development of premature senescence in fibroblasts. This pathway is active in normal epithelial cells, which express caveolin-1, but not in MCF-7 breast cancer cells, which do not express caveolin-1 and undergo apoptosis after oxidative stress. Reexpression of caveolin-1 in MCF-7 cells prevents apoptosis and promotes senescence after oxidative stress. We propose caveolin-1 as a switcher that controls senescent and apoptotic cellular programs in breast cancer cells subjected to oxidative stress.

promoter sequences. Endogenous antioxidants such as reduced glutathione decrease with age (48), whereas caveolin-1 protein expression has been shown to increase with age (49). Thus, we may speculate that up-regulation of caveolin-1 occurring with the aging process may be due, in part, to the accumulation of oxidants and the reduction of endogenous antioxidants.

What signaling molecule is upstream of Sp1? Our data indicate that p38 MAPK is activated by oxidative stress and activation of the p38 MAPK pathway is required for oxidative stress–induced Sp1-mediated transcription of the caveolin-1 promoter. We also show that inhibition of p38 MAPK prevented the Sp1-mediated up-regulation of caveolin-1 protein expression and induction of premature senescence upon oxidative stress. These results are schematically summarized in Fig. 6*D*. Because up-regulation of caveolin-1 expression is required for stress-induced premature senescence in fibroblasts (33), these findings suggest that prevention of up-regulation of caveolin-1 may represent the molecular mechanism through which inhibition of p38 MAPK negatively regulates cellular senescence.

Because we have previously shown that the maintenance of caveolin-1 expression at low levels was sufficient to prevent stress-induced premature senescence (33), it is possible to speculate that a combination of (*i*) prevention of up-regulation of caveolin-1 expression and (*ii*) p38 MAPK inhibitors may represent a potential novel approach to prevent/delay aging and degenerative diseases that have been associated to cellular stress.

In recent years, several independent lines of evidence have emerged suggesting that caveolin-1 may function as a "tumor suppressor protein" in mammalian cells. For example, caveolin-1 protein expression has been shown to be absent in several transformed cell lines derived from human mammary carcinomas, including MCF-7, MT-1, ZR-75-1, T47D, MDA-MB-361, and MDA-MB-474 (32). In addition, caveolin-1 mRNA and protein expression are lost or reduced during cell transformation by activated oncogenes, such as v-*Abl* and H-*ras* (G12V); caveolae are absent from these cell lines. In addition, the human caveolin-1 gene is localized to a suspected tumor suppressor locus (*D7S522*; 7q31.1), a known fragile site (FRA7G) that is deleted in many types of cancer (23, 50–52).

Here, we also show that oxidative stress activated the p38 MAPK–mediated up-regulation of caveolin-1 expression and induction of premature senescence in normal mammary epithelial cells, suggesting that this pathway is not cell type specific but is active both in fibroblasts and epithelial cells. In contrast, activation of this pathway by oxidants was repressed in MCF-7 breast cancer cells. Oxidants are also known to induce apoptosis. Interestingly, oxidative stress induced apoptosis in caveolin-1-negative MCF-7 cells, but not premature senescence. Lack of caveolin-1 is necessary to drive the apoptotic response to oxidants in these cancer cells, as shown by prevention of apoptosis when caveolin-1 was reintroduced in MCF-7 cells. In addition to preventing apoptosis, caveolin-1 reexpression in MCF-7 cells turned on a senescent response after oxidative stress. We conclude that caveolin-1 controls the apoptotic to senescent program switch in breast cancer cells subjected to oxidative stress (Fig. 6*D*). These results are consistent with the previously shown inhibitory effect of caveolin-1 overexpression in MCF-7 cells on anchorage-independent growth, anoikis, and invasiveness (53).

Tumor development is initiated by a multiplicity of genetic abnormalities. Moreover, tumor cells need to escape barriers that limit uncontrolled cell proliferation. One of these barriers is represented by cellular senescence. Cancer cells need to overcome this obstacle to produce a clinically relevant tumor mass. For these reasons, cellular senescence represents a natural tumor suppressor mechanism (28, 42, 54–58). We enforce in this report the concept that caveolin-1 gene transcription and caveolin-1 protein expression are critical for the development of the senescent phenotype. Thus, cellular senescence may represent one of the molecular mechanisms through which caveolin-1 acts as a tumor suppressor protein.

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Oxidative Stress Induces Premature Senescence by Stimulating Caveolin-1 Gene Transcription through p38 Mitogen-Activated Protein Kinase/Sp1–Mediated Activation of Two GC-Rich Promoter Elements

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