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Regulation of Lung Cancer Cell Migration and Invasion by Reactive Oxygen Species and Caveolin-1*

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The acquired capability of tumor cells to migrate and invade neighboring tissues is associated with high metastatic potential and advanced stage of cancers. Recently, signaling molecules such as reactive oxygen species (ROS) and caveolin-1 (Cav-1) have been implicated in the aggressive behavior of cancer cells. However, the roles of specific ROS in cancer cell migration and Cav-1 regulation are unclear. We demonstrate here that Cav-1 plays an important role in the migration and invasion of human lung carcinoma H460 cells and that these effects are differentially regulated by cellular ROS. Using various known inhibitors and donors of ROS, we found that different ROS have different effects on Cav-1 expression and cell migration and invasion. Superoxide anion and hydrogen peroxide down-regulated Cav-1 expression and inhibited cell migration and invasion, whereas hydroxyl radical up-regulated the Cav-1 expression and promoted cell migration and invasion. The down-regulating effect of superoxide anion and hydrogen peroxide on Cav-1 is mediated through a transcription-independent mechanism that involves protein degradation via the ubiquitin-proteasome pathway. These results indicate the essential role of different ROS in cancer cell motility and through Cav-1 expression, which may provide a key mechanism controlling tumor progression and metastasis. The up-regulation of Cav-1 and cell motility by hydroxyl free radical suggests an important role of this ROS as a positive regulator of tumor progression.

Cancer cell migration and invasion are initial steps in metastasis that is a primary cause of cancer-related death. During metastasis, primary tumor cells migrate and invade neighboring tissues and enter the circulation to establish new or secondary tumor sites (1–3). Increasing evidence suggests that signaling molecules presenting in the tumor microenvironment have a significant impact on the migratory properties of cancer cells (4, 5). For example, increased ROS[4] in the tumor microenvironment has been associated with increased aggressiveness of cancer cells (6, 7). Although several studies have investigated the effects of ROS on cell migration and invasion, variable results have been reported depending on the type of ROS, dose, and production site, as well as the tissue type of cells (8–12). Several mechanisms of ROS regulation of cancer cell migration have been proposed; most of these involve alterations of cellular cytoskeleton and adhesion molecules. For instance, ROS have been reported to regulate integrin (13, 14), small GTPase Rho family proteins (15, 16), focal contact-forming proteins (13, 17), and extracellular matrix-degrading enzymes such as matrix metalloproteinases (14, 18, 19).

Caveolin-1 (Cav-1) is an essential structural protein component of the plasma membrane microdomains called caveolae. It has been shown to function in vesicular trafficking, signal transduction, and tumor progression. Cav-1 interacts with several signaling molecules, including Ha-Ras, Src family tyrosine kinases, G protein α subunits, and protein kinase C (20–23). Increased expression of Cav-1 has been observed in lung adenocarcinoma and prostate cancer, which are associated with their invasiveness (24–26). In lung carcinoma, the elevated Cav-1 expression is also associated with an increase in metastatic capacity and poor survival of patients (26–28). On the other hand, Cav-1 has been shown to have a suppressive effect on pancreatic and breast cancer cell motility (29–31). Thus, the role of Cav-1 in cancer cell migration and metastasis remains unclear and appears to be cell type-dependent.

Increased oxidative stress and ROS production have also been associated with many human metastatic tumors, including the lung (32, 33), breast (34), prostate (35), colon (36), and ovary (37). ROS such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) have been shown to be up-regulated in the tumor microenvironment and have been suggested to play a role in tumor progression and metastasis (5, 7). Although ROS are likely to affect cancer cell motility and invasiveness through multiple mechanisms, Cav-1 may play a key role in this process because Cav-1 is known to be abnormally regulated in invasive tumors and to play a role in

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[4] The abbreviations used are: ROS, reactive oxygen species; Cav-1, caveolin-1; H_2O_2, hydrogen peroxide; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; $FeSO_4$, ferrous sulfate; NaFM, sodium formate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CAT, catalase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide.

cancer cell migration (24–28). Cav-1 is also subject to regulation by ROS (38, 39), but the underlying mechanism of regulation and the specific ROS involved are unclear. In this study, we investigated the roles of specific ROS, including $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$ in Cav-1 expression and cell migration, and determined the mechanisms of regulation in human lung carcinoma H460 cells. The following specific points are addressed: (*a*) whether Cav-1 plays a role in the cell migration and invasion; (*b*) whether Cav-1 is regulated by ROS and if so by what mechanism; and (*c*) what specific ROS are involved and how they affect cell migration and invasion.

MATERIALS AND METHODS

Cells and Reagents—Human lung cancer epithelial H460 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 environment at 37 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), Hoechst 33342, hydrogen peroxide, catalase, ferrous sulfate heptahydrate, sodium formate, deferoxamine, lactacystin, type IV collagen, and laminin were obtained from Sigma. Mn(III)tetrakis(4-benzoic acid)-porphyrin chloride (MnTBAP) and concanamycin A were obtained from Calbiochem. Antibodies for Cav-1, ubiquitin, β-actin, peroxidase-labeled secondary antibodies, and protein-G agarose were obtained from Abcam Inc. (Cambridge, MA). Antibody for phospho-Akt (Ser-473) was from Cell Signaling Technology, Inc. (Beverly, MA).

Generation of Stable Cav-1-overexpressing Cells—H460 cells were seeded in a 6-well plate until they reached 60% confluence. The cells were then transfected with caveolin-1 plasmid (pEX_Cav-1-YFP) (ATCC) or control plasmid (pcDNA3) using Lipofectamine reagent (Invitrogen) in culture medium in the absence of serum. After 12 h of incubation, the medium was replaced with complete culture medium containing 10% FBS. Approximately 36 h after the beginning of the transfection, the cells were trypsinized and plated onto 75-ml culture flasks and cultured for 28 days with neomycin-containing medium (800 μg/ml). The pooled stable transfectant was identified by Western blotting of Cav-1 and was cultured in neomycin-free RPMI 1640 medium for at least two passages before each experiment.

Inhibition of Cav-1 by RNA Interference—Lentiviral transduction particles carrying short hairpin RNA (shRNA) sequence against human Cav-1 (5′-CCGGGACGTGGTCAAGATTGACTTTCTCGAGAAAGTCAATCTTGACCACGTCTTTTT-3′) and control nontarget sequence (5′-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3′) were used to knock down Cav-1 expression in H460 cells. The viral vectors were obtained commercially from Sigma (accession numbers NM_001753 and SHC002V) and were used according to the manufacturer's instruction. Briefly, cells were seeded in 6-well plates (5×10^5/well) and incubated with Cav-1 shRNA lentiviral particles or control particles at the multiplicity of infection of 1.5 in the presence of hexadimethrine bromide (8 μg/ml) for 36 h. Transfected cells were analyzed for Cav-1 by Western blotting prior to use.

Cytotoxicity Assay—Cell viability was determined by MTT assay. After specific treatments, cells in 96-well plates were incubated with 500 μg/ml of MTT for 4 h at 37 °C. The intensity of formazan product was measured at 550 nm using a microplate reader. Absorbance ratio of treated to nontreated control cells was calculated and presented as relative cell viability.

ROS Detection—Cellular ROS was determined by flow cytometry using H_2DCF-DA as a fluorescent probe and by electron spin resonance (ESR) spectroscopy using 5,5-dimethyl-1-pyrroline *N*-oxide as a spin-trapping agent. For flow cytometric measurements, cells were incubated with H_2DCF-DA (10 μM) for 30 min at 37 °C, after which they were washed and resuspended in phosphate-buffered saline (PBS) and analyzed for fluorescence intensity using a 485-nm excitation beam and a 538-nm bandpass filter (FACSort, BD Biosciences). The mean fluorescence intensity was quantified by CellQuest software (BD Biosciences) analysis of the recorded histograms.

For ESR measurements, cells were incubated with DMPO (100 mM) for 10 min at 37 °C in the presence or absence of ROS modulators. ESR signals were measured using a Bruker EMX spectrometer (Bruker Instruments, Billerica, MA) and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate and 1,1-diphenyl-2-picrylhydrazyl as reference standards. An Acquisit program (Bruker Instruments) was used for data acquisition and analysis.

Migration Assay—Cell migration was determined by wound or scratch assay. A monolayer of cells was cultured in 24-well plate, and then a wound space was made with a 1-mm width tip. After rinsing with PBS, the cell monolayers were treated with specific ROS modulators and allowed to migrate for 24 h. Micrographs were taken under a phase contrast microscope (×100) (Olympus DP70, Melville, NY), and wound spaces were measured from 10 random fields of view using an Olympus DP controller software. Quantitative analysis of cell migration was performed by using an average wound space from those random fields of view, and the percentage of change in the wound space was calculated using the following formula: % change = (average space at time 0 h) − (average space at time 24 h)/(average space at time 0 h) × 100. Relative cell migration was calculated by dividing the percentage change in the wound space of treated cells by that of the control cells in each experiment.

Invasion Assay—Invasion assay was performed using a 24-well Transwell unit with polycarbonate (PVDF) filters (8 μm pore size). The membrane was coated with a mixture of laminin (50 μg/ml), type IV collagen (50 μg/ml), and gelatin solution (2 mg/ml in 10 mM glacial acetic acid) (25). Cells at the density of 2×10^4 cells per well were seeded into the upper chamber of the Transwell unit in serum-free medium. The lower chamber of the unit was added with a medium containing 10% FBS. After incubation with specific test agents for

24 h at 37 °C, the medium in the upper chamber was sucked out, and the cells on the upper side of membrane were removed with a cotton swab. Cells that invaded to the underside of the membrane were stained with 10 μg/ml Hoechst 33342 for 10 min and visualized and scored under a fluorescence microscope.

Western Blot Analysis—After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Applied Science) at 4 °C for 20 min. Cell lysates were collected and determined for protein content using the Bradford method (Bio-Rad). Proteins (40 μg) were resolved under denaturing conditions by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM sodium chloride, 0.05% Tween 20) and incubated with appropriate primary antibodies at 4 °C for 10 h. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-labeled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were then detected by enhanced chemiluminescence detection system (Amersham Biosciences) and quantified using analyst/PC densitometry software (Bio-Rad).

Immunoprecipitation—After specific treatments, cells were washed with PBS and lysed in lysis buffer at 4 °C for 20 min. Cell lysates were collected and determined for protein content using the Bradford method. Lysate proteins (60 μg) were incubated with Cav-1 antibody for 14 h at 4 °C, followed by a 4-h incubation with protein G-conjugated agarose at 4 °C. The immune complexes were washed six times with cold lysis buffer and resuspended in 2× Laemmli sample buffer. The immune complexes were separated by 10% SDS-PAGE and analyzed by Western blotting as described.

Quantitative Real Time PCR—Total RNA was extracted with TRIzol reagent (Invitrogen). One microgram of extracted RNA was reverse-transcribed in a 100-μl reaction mixture containing 500 μM dNTP, 125 units of MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA), 40 units of RNase inhibitor, 2.5 μM oligo(dT), 1× TaqMan reverse transcriptase buffer, and 5 mM $MgCl_2$ at 48 °C for 40 min. The primers used in this study were designed using Primer Express software (Applied Biosystems) as follows: Cav-1 (AI878826), forward 5′-CGAGAAGCAAGTGTACGACGC-3′ and reverse 5′-ACCACGTCATCGTTGAGGTG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′. Amplification was performed at the following cycling conditions: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A SYBR Green PCR master mix (Applied Biosystems) was used with 1 ng of cDNA and with 100–400 nM primers. A negative control without any cDNA template was run with every assay. All PCRs were performed by using ABI PRISM 7900 sequence detection system (Applied Biosystems). Relative mRNA levels were determined by using the comparative

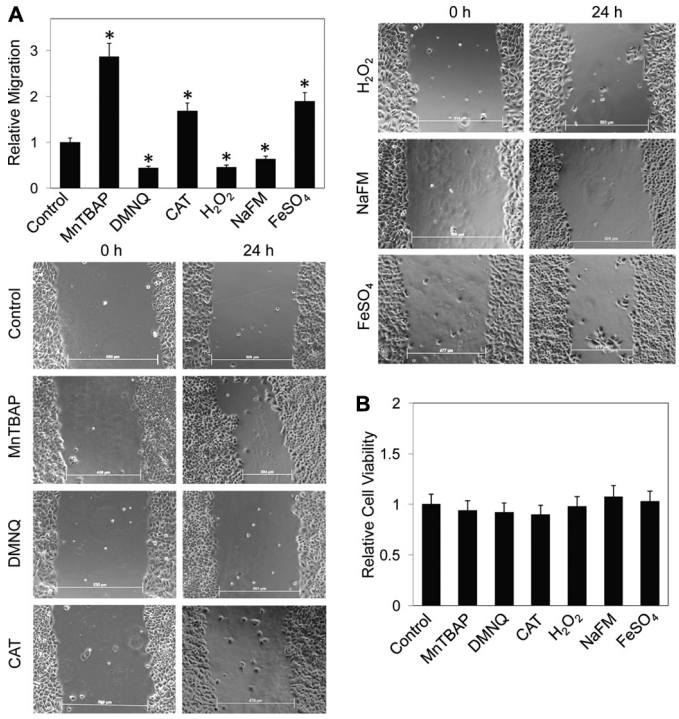

FIGURE 1. **ROS regulate migration of human lung epithelial H460 cells.** *A*, confluent monolayers of H460 cells were wounded, and the cells were allowed to migrate for 24 h in the presence or absence of various ROS modulators, including MnTBAP (50 μM), DMNQ (5 μM), CAT (7,500 units/ml), H_2O_2 (100 μM), NaFM (5 mM), and $FeSO_4$ (50 μM). Wound space was visualized under a phase contrast microscope and analyzed by comparing the relative change in wound space of the treated over nontreated cell monolayers. Representative micrographs from four independent experiments are shown. *B*, effect of ROS modulators on cell viability. Cell monolayers were similarly treated with the indicated concentrations of ROS modulators and analyzed for cell viability after 24 h by MTT assay. Data are the mean ± S.D. ($n = 4$). *, $p < 0.05$ *versus* nontreated control.

CT (threshold cycle) method (40), where the caveolin-1 target is normalized to the control and compared with a reference sample (assigned a relative value of 1) by the equation: $2^{-\Delta\Delta CT}$.

Statistical Analysis—Data were represented as the means ± S.D. from three or more independent experiments. Statistical analysis was performed by Student's *t* test at a significance level of $p < 0.05$.

RESULTS

Differential Effect of ROS on Cell Migration—ROS have been shown to be involved in several cellular migratory processes, including wound repair, metastasis, and angiogenesis (8–12, 41, 42). However, the roles of specific ROS and regulatory mechanisms are not well understood. We tested whether ROS play a role in the migration of lung carcinoma H460 cells and determined the specific ROS involved. Cells were treated with various known inducers and scavengers of ROS, and their effect on cell migration was determined by wound migration assay. Fig. 1*A* shows that treatment of the cells with MnTBAP, a superoxide dismutase mimetic and scavenger of $O_2^{\cdot-}$, stimulated the migration of cells across the wound space, whereas treatment of the cells with DMNQ, a known inducer of $O_2^{\cdot-}$ (43, 44), had an opposite effect. Likewise, treatment of the cells with catalase (CAT) (H_2O_2 scavenger) promoted cell

migration, whereas treatment with H_2O_2 inhibited the migration. These results indicate the inhibitory role of $O_2^{\cdot-}$ and H_2O_2 in the migration of H460 cells during wound healing. In contrast to the above findings, treatment of the cells with sodium formate (NaFM), a known $OH^{\cdot}$ scavenger (45, 46), inhibited the migration, although the $OH^{\cdot}$ generator ferrous sulfate ($FeSO_4$) promoted this effect. These results suggest the differential roles of ROS in the regulation of cancer cell migration with $OH^{\cdot}$ playing a promoting role and $O_2^{\cdot-}$ and H_2O_2 having an inhibitory role.

To ensure that the observed inhibitory and stimulating effects of ROS modulators were not caused by a cytotoxic effect, cells were treated with the same concentrations of the test modulators, and their effect on cell viability was determined by MTT assay. The results show that none of the treatments caused a significant effect on cell viability as compared with nontreated control (Fig. 1*B*).

Detection of Cellular ROS—To provide supporting evidence for the role of ROS in cell migration, cellular ROS in response to the above treatments were determined by flow cytometry and ESR. Flow cytometric analysis was performed using H_2DCF-DA as a fluorescent probe. H_2DCF-DA is a general oxidative probe that can detect multiple ROS. The probe enters the cells and is cleaved by cellular esterases to yield a nonfluorescent product, dichlorofluorescin, which is trapped inside the cells. Upon oxidation by ROS, dichlorofluorescein is converted to the fluorescent product dichlorofluorescein, which is detected by flow cytometry. Fig. 2*A* shows that the ROS inducers DMNQ, H_2O_2, and $FeSO_4$ were able to increase the cellular DCF fluorescence intensity over control levels, whereas the ROS scavengers MnTBAP, CAT, and NaFM decreased the fluorescence intensity, indicating multiple ROS generation and scavenging by the treatments.

To confirm the formation of ROS in the treated cells, ESR measurements were performed using DMPO as a spin-trapping agent to aid the detection of short lived oxygen free radicals. Fig. 2*B* shows a typical ESR spectrum generated by incubating H460 cells in culture medium with DMPO. A weak ESR signal consisting of a 1:2:2:1 quartet, which is a characteristic of DMPO-$OH^{\cdot}$ adduct, was observed, indicating the formation of $OH^{\cdot}$ radicals. Addition of the Fenton catalyst and $OH^{\cdot}$ generator $FeSO_4$ to the cells intensified this signal, supporting the generation of $OH^{\cdot}$ radicals. Addition of the $OH^{\cdot}$ scavenger NaFM to the $FeSO_4$-treated cells inhibited the ESR signal, indicating the specificity of $OH^{\cdot}$ detection and their scavenging by NaFM under the test conditions. Neither H_2O_2 nor $O_2^{\cdot-}$ was detectable in this study because the former is not a free radical, and the latter does not form a stable adduct with the spin-trapping agent DMPO.

Cav-1 Promotes Cell Migration and Invasion—Cav-1 has been shown to modulate cell migration in different cell types (20–25, 29–31). We tested whether Cav-1 can regulate the migration and invasion of lung carcinoma H460 cells. The cells were stably transfected with Cav-1 or control plasmid, and their effects on Cav-1 expression, cell migration, and invasion were determined. Western blot analysis of Cav-1 expression shows a substantial increase in the expression in Cav-1-transfected cells as compared with vector-transfected

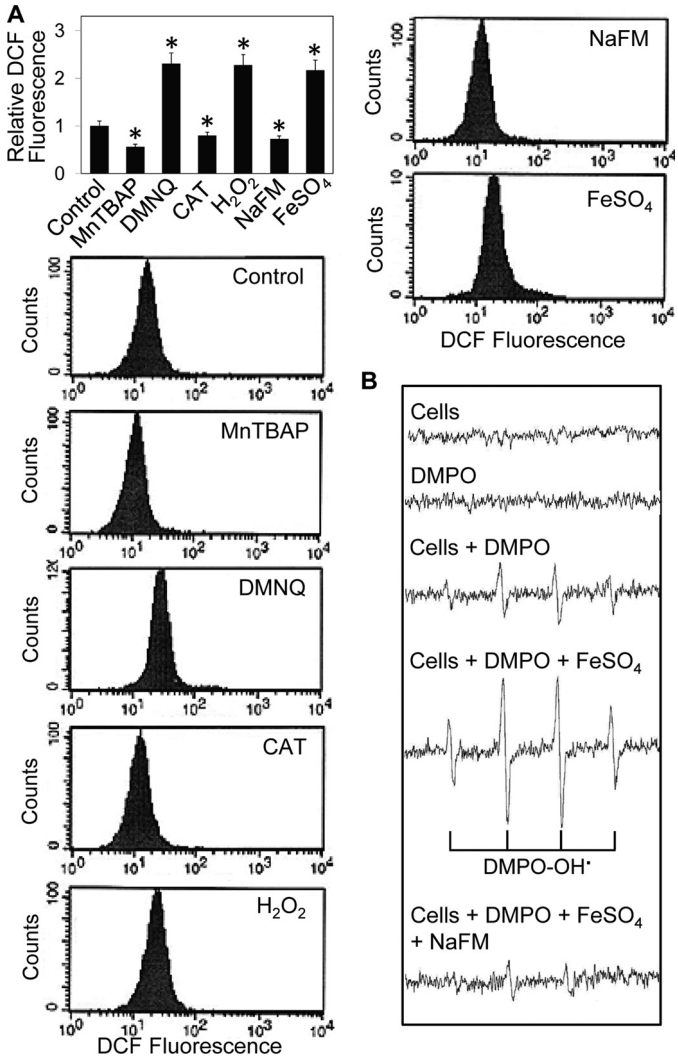

FIGURE 2. **Effect of ROS modulators on cellular ROS.** *A*, H460 cells were treated with the indicated concentrations of ROS modulators as shown in Fig. 1 and analyzed for ROS levels by flow cytometry using H_2DCF-DA as a fluorescent probe. Data shown are relative fluorescence intensities over control level determined at 2 h post-treatment. *B*, ESR detection of ROS. H460 cells (1×10^6 cells/ml) were incubated in culture medium containing the spin trapper DMPO (100 mM) with or without $FeSO_4$ (50 μM) and NaFM (5 mM). ESR spectra were recorded 10 min after the addition of the test agents. The spectrometer settings were as follows: receiver gain at 2.5×10^4, time constants at 0.04 s, modulation amplitude at 1.0 G, scan time at 42 s, magnetic field at 3475 ± 100 G. Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* nontreated control.

control (Fig. 3*A*). Wound migration assay shows that the Cav-1-transfected cells exhibited an increase in migratory activity as compared with control-transfected cells (Fig. 3*B*). Transwell invasion assay similarly indicates an increase in cell invasiveness in the Cav-1-overexpressing cells as compared with control-transfected cells (Fig. 3*C*).

To confirm the role of Cav-1 in cell migration and invasion, Cav-1 expression was inhibited by RNA interference using shRNA against Cav-1 (shCav-1). H460 cells were treated with shCav-1 viral particles or control shRNA (shCon) particles, and their effects on cell migration and invasion were determined. Fig. 3*D* shows that Cav-1 expression was substantially reduced in shCav-1-treated cells as compared with shCon-treated cells (*left panel*). Rescue experiment was performed

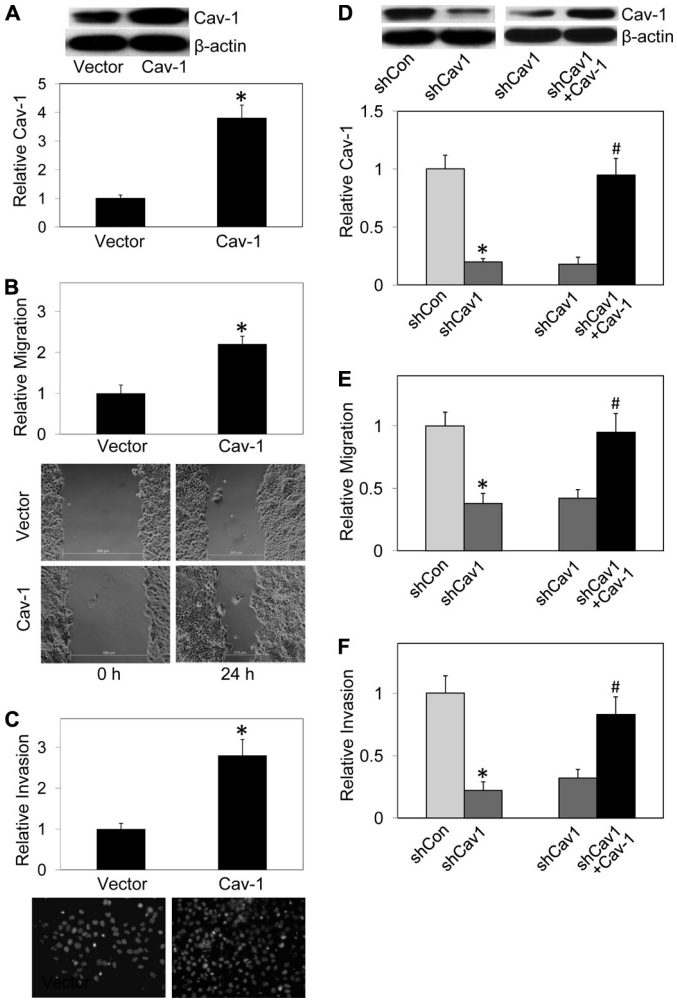

FIGURE 3. **Effects of Cav-1 overexpression and knockdown on cell migration and invasion.** H460 cells were stably transfected with Cav-1 or control plasmid as described under "Materials and Methods." *A*, Cav-1 expression in the control and Cav-1-transfected cells was determined by Western blotting. Cell extracts were prepared and separated on 10% SDS-polyacrylamide gels, transferred, and probed with Cav-1 antibody. *β*-Actin was used as a loading control. *B*, effect of Cav-1 overexpression on cell migration. Cav-1 and control-transfected cells were cultured in 24-well plates and analyzed for cell migration at 24 h by wound assay. *C*, effect of Cav-1 overexpression on cell invasion. Cav-1 and control-transfected cells were added to extracellular matrix-coated inserts in a Transwell chamber and incubated for 24 h. Invading cells were counted under a fluorescence microscope after staining with Hoechst 33342, and the average number of cells was scored in each case. *D–F*, Cav-1 knockdown experiments were performed using H460 cells treated with Cav-1 shRNA (*shCav-1*) viral particles or control shRNA (*shCon*) particles as described under "Materials and Methods." *D*, Cav-1 expression in shCav-1 and shCon-treated cells determined by Western blotting at 36 h post-treatment (*left panel*). Rescue experiment was performed in shCav-1-treated cells by transfecting the cells with Cav-1 plasmid as described above and analyzed for Cav-1 expression by Western blotting (*right panel*). *E* and *F*, migration and invasion of shCon, shCav-1, and rescued cells determined by wound and Transwell assays, respectively. Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control transfection; #, $p < 0.05$ *versus* shCav1 control.

on the shCav-1 knockdown cells to exclude the off-target effect of shCav-1 (Fig. 3*D, right panel*). Fig. 3, *E* and *F,* shows that shCav-1 cells exhibited reduced migratory and invasive activities as compared with shCon cells and that overexpression of Cav-1 in these cells reversed the migratory and invasive activities. These results support the promoting role of Cav-1 in migration and invasion of H460 cells.

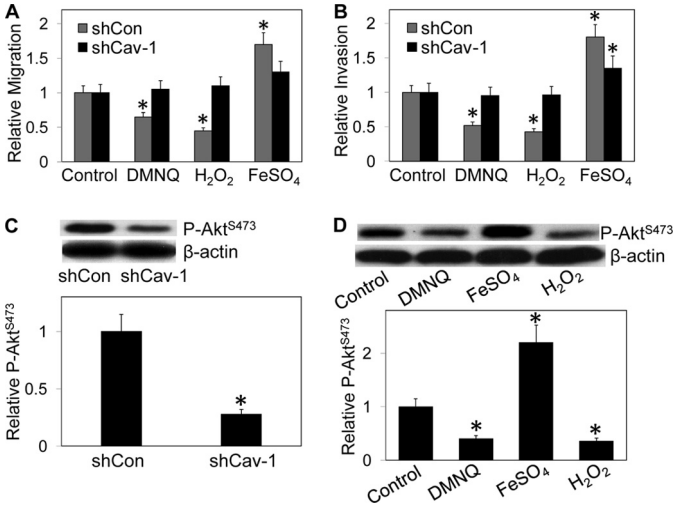

FIGURE 4. **Effects of Cav-1 knockdown on ROS-modulated cell migration, invasion, and Akt activity.** *A* and *B*, shCav-1 and shCon cells were treated with DMNQ (5 μM), H_2O_2 (100 μM), and $FeSO_4$ (50 μM) for 24 h and analyzed for cell migration and invasion. *C*, shCav-1 and shCon cells were analyzed for Akt phosphorylation by Western blotting. Blots were probed with phospho-Akt Ser-473 antibody and reprobed with *β*-actin antibody. *D*, H460 cells were treated with DMNQ (5 μM), H_2O_2 (100 μM), and $FeSO_4$ (50 μM) and analyzed for Akt phosphorylation at 6 h post-treatment. Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control cells.

To determine whether the effects of ROS on cell migration and invasion are dependent on Cav-1 expression, shCav-1 and shCon cells were treated with DMNQ, H_2O_2, and $FeSO_4$, and their effects on cell migration and invasion were determined. Fig. 4, *A* and *B,* shows that DMNQ and H_2O_2 inhibited cell migration and invasion in shCon cells but not in shCav-1 cells. $FeSO_4$ increased cell migration and invasion in shCon cells but had minimal effects in shCav-1 cells. These results indicate the role of Cav-1 in the ROS effects on cell migration and invasion.

Cav-1 has been shown to regulate cell migration through a PI3K/Akt-dependent mechanism (47–49). We tested whether knockdown of Cav-1 affects Akt activity by analyzing phosphorylated Akt (pAkt) levels in shCav-1 and shCon cells. Fig. 4*C* shows that the pAkt level was substantially reduced in shCav-1 cells as compared with shCon cells. This result is consistent with our previous report showing the induction of Akt activation by Cav-1 overexpression (50). We also investigated Akt activity in H460 cells in response to specific ROS. Fig. 4*D* shows that DMNQ and H_2O_2 caused a decrease in pAkt level, whereas $FeSO_4$ promoted it. These results are consistent with the observed effects of specific ROS on Cav-1 expression and cell motility, supporting the role of Cav-1 in the migratory process through Akt signaling.

ROS Regulate Cav-1 Expression—Cav-1 may be regulated by ROS, which may represent a key mechanism of cell migratory regulation by ROS. To test this possibility, cells were treated with various ROS modulators, and their effect on Cav-1 expression was determined by Western blotting. Fig. 5*A* shows that treatment of the cells with DMNQ or H_2O_2 substantially down-regulated the expression of Cav-1, whereas treatment with $FeSO_4$ up-regulated the expression. Consistent with the inhibitory role of O_2^- and H_2O_2, the scavengers of these ROS (MnTBAP and CAT, respectively) pro-

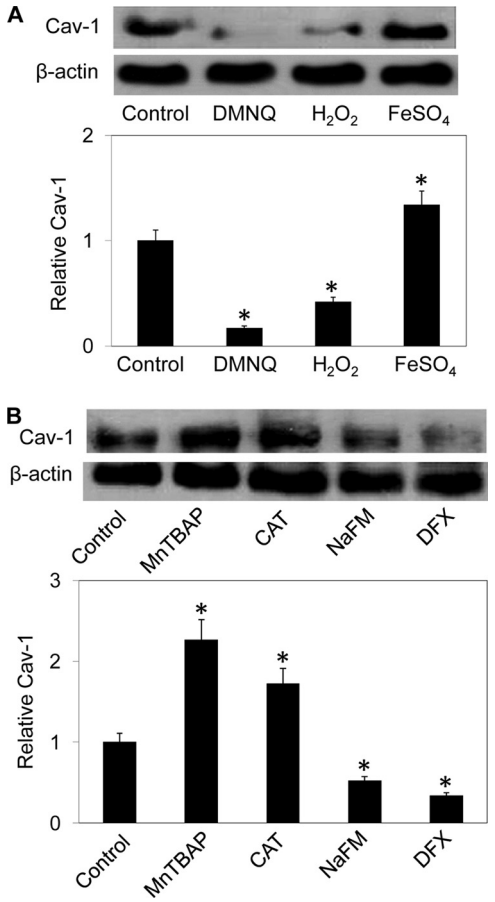

FIGURE 5. **ROS regulate Cav-1 expression.** *A*, H460 cells were treated with various ROS generators, including DMNQ (5 μM), H_2O_2 (100 μM), and $FeSO_4$ (50 μM) for 24 h, and cell lysates were prepared and analyzed for Cav-1 expression by Western blotting. *B*, cells were treated with various ROS scavengers or inhibitors, including MnTBAP (50 μM), CAT (7,500 units/ml), NaFM (5 mM), and deferoxamine (*DFX*) (0.5 mM), and Cav-1 expression was determined after 24 h. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and mean data from three independent experiments (one of which is shown here) were normalized to the result obtained in control cells. Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* nontreated control.

moted the Cav-1 expression (Fig. 5*B*). In contrast, the $OH^{\bullet}$ scavenger NaFM inhibited the expression, supporting the positive regulatory role of $OH^{\bullet}$ in Cav-1 expression. This latter result was confirmed by the observation that deferoxamine, a known metal chelator and inhibitor of $OH^{\bullet}$, also inhibited Cav-1 expression (Fig. 5*B*).

Role of Specific ROS in Cell Migration, Invasion, and Cav-1 Expression—To determine further the relationship between Cav-1 expression and cell motility regulation by different ROS, cells were treated with various concentrations of ROS modulators, and their effects on cell migration, invasion, and Cav-1 expression were determined. Fig. 6, *A* and *D*, shows that DMNQ caused a dose-dependent and parallel decrease in both cell migration and Cav-1 expression. Addition of MnTBAP reversed both of these effects of DMNQ, indicating the role of $O_2^{\bullet-}$ in the processes and their association. Similar to DMNQ, H_2O_2 caused a dose-dependent and concomitant decrease in cell migration (Fig. 6*B*) and Cav-1 expression (Fig. 6*E*), both of which were inhibited by catalase. In contrast to $O_2^{\bullet-}$ and H_2O_2, $OH^{\bullet}$ promoted cell migration (Fig. 6*C*) and Cav-1 expression (Fig. 6*F*) as indicated by their positive responses to $FeSO_4$ treatment and their inhibition by NaFM co-treatment, which were shown to induce and inhibit, respectively, the formation of $OH^{\bullet}$ radicals (Fig. 2*B*). Together, these results indicate the positive regulatory role of $OH^{\bullet}$ and the opposing role of $O_2^{\bullet-}$ and H_2O_2 in cell migration through Cav-1 expression. A similar finding was observed with regard to the role of different ROS in cell invasion (Fig. 6*G*).

Superoxide and Hydrogen Peroxide Down-regulate Cav-1 by Ubiquitin-Proteasomal Degradation—To investigate the mechanism of Cav-1 down-regulation by $O_2^{\bullet-}$ and H_2O_2, cells were treated with DMNQ and H_2O_2 and analyzed for Cav-1 mRNA expression by quantitative real time RT-PCR. Although causing a substantial decrease in Cav-1 protein expression (Fig. 6*A*), treatment of the cells with DMNQ and H_2O_2 did not cause a corresponding decrease in Cav-1 mRNA expression (Fig. 7*A*). In fact, a slight increase was observed, possibly due to stress induction. Thus, $O_2^{\bullet-}$ and H_2O_2 appear to regulate Cav-1 protein expression through a transcription-independent mechanism.

Because many proteins are regulated by ROS through protein degradation, we tested the potential role of degradation in ROS-mediated Cav-1 down-regulation. Degradation of endogenous proteins normally occurs through two major pathways, proteasomal and lysosomal. We therefore tested the effect of proteasome inhibitors, lactacystin and MG132, and the lysosome inhibitor, concanamycin A, on ROS-induced Cav-1 down-regulation. Fig. 7, *B* and *C*, shows that lactacystin and MG132, but not concanamycin A, inhibited Cav-1 down-regulation by DMNQ and H_2O_2, suggesting proteasomal degradation as a primary mechanism of Cav-1 down-regulation by ROS.

Because proteins are targeted for proteasomal degradation via ubiquitination, we tested whether ROS modulators induce ubiquitination of Cav-1 protein. Cells were treated with DMNQ and H_2O_2, and cell lysates were prepared and immunoprecipitated by anti-Cav-1 antibody. The resulting immune complexes were then analyzed for ubiquitination by Western blots using anti-ubiquitin antibody. Fig. 7, *D* and *E*, shows that both DMNQ and H_2O_2 were able to induce ubiquitination of Cav-1 and that these effects were inhibited by co-treating the cells with MnTBAP and catalase, respectively. Together, these results indicate ROS-mediated down-regulation of Cav-1 through ubiquitin-proteasomal degradation.

ROS Regulate Migration and Invasion of Cav-1-overexpressing H460 Cells and Melanoma G361 Cells—To substantiate the role of ROS in cell migration and invasion, experiments were performed in Cav-1 overexpressing H460 cells described earlier and in melanoma G361 cells to determine the cell type-specific effects of ROS. The cells were treated with the ROS modulators DMNQ, H_2O_2, and $FeSO_4$, and their effects on cell migration and invasion were examined. The results show that DMNQ and H_2O_2 had similar inhibitory effects on cell migration and invasion in these cells, although $FeSO_4$ had opposite effects (Fig. 8). These results are consistent with the earlier findings in H460 cells (Fig. 6) and suggest the generality of the observed ROS effects on cell migration and invasion.

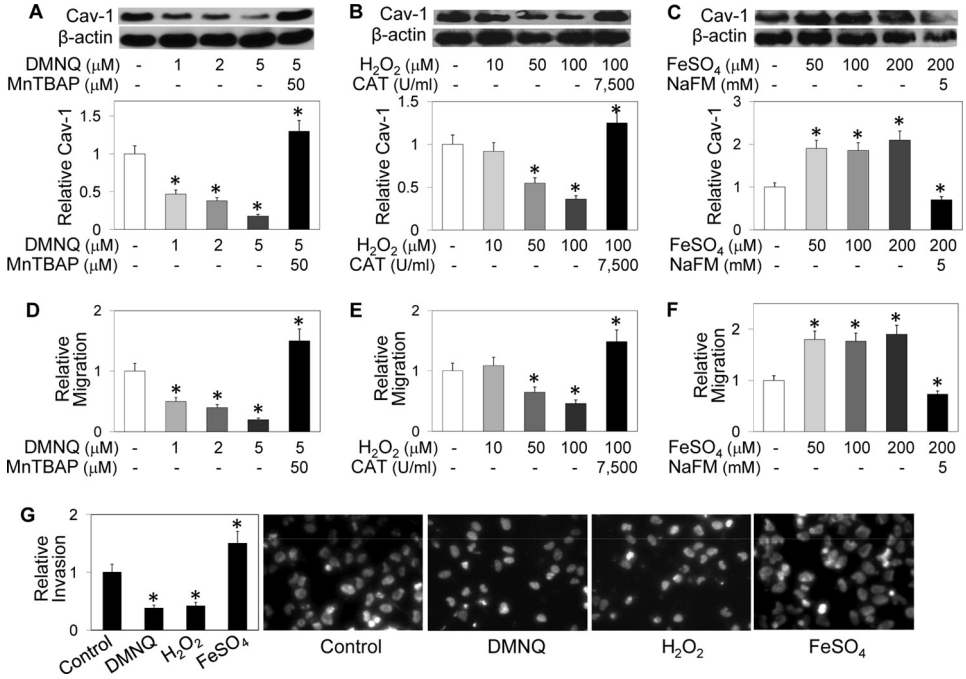

FIGURE 6. **Differential effects of ROS on Cav-1 expression, cell migration, and invasion.** Western blot analysis is shown of Cav-1 expression in H460 cells treated with the indicated concentrations of DMNQ and MnTBAP (*A*), H_2O_2 and CAT (*B*), and $FeSO_4$ and NaFM (*C*) for 24 h. Cell migration was determined 24 h after the treatment with DMNQ and MnTBAP(*D*), H_2O_2 and CAT (*E*), and $FeSO_4$ and NaFM (*F*). Cell invasion was determined 24 h after the treatment with DMNQ (5 μM), H_2O_2 (100 μM), and $FeSO_4$ (50 μM) (*G*). Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* nontreated control.

DISCUSSION

The role of ROS in the regulation of cell migration and invasion has been described (5, 7), but the role of specific ROS and their regulatory mechanisms have not been well investigated. We report here that different ROS have different effects on cell migration and invasion in human lung carcinoma H460 cells. $O_2^{\cdot-}$ and H_2O_2 suppress the migration and invasion of the cells, whereas $OH^{\cdot}$ promotes the cell motility activities. Because several ROS are generated during oxidative stress, which has been linked to tumor progression, the results of this study further indicate that depending on the type and abundance of specific ROS generated, oxidative stress conditions may promote or suppress tumor progression by affecting cell migration and invasion.

This study also demonstrates the role of Cav-1 as a key target of ROS regulation of cell motility. Cav-1 has been shown to be involved in cancer cell motility and tumor progression (21, 29–31, 51). However, its precise role and regulatory mechanisms are still unclear as both promoting and inhibitory roles of Cav-1 have been reported. In pancreatic cancer cells, Cav-1 was shown to inhibit cell migration and invasion through the inactivation of RhoC GTPase and ERK-metalloproteinase signaling pathways (29, 52). A similar inhibitory effect of Cav-1 was observed in breast cancer MTLn3 and MCF-7 cells (30, 53). In contrast, Cav-1 has been reported to promote lung cancer cell invasion by mediating filopodia formation (25). Furthermore, Cav-1 expression is associated with the tumor grade and metastasis of non-small cell lung cancer (27, 54). Consistent with the tumor-promoting role of Cav-1, we found that Cav-1 promotes the migration and invasion of non-small cell lung cancer H460 cells. The function of Cav-1 is closely associated with its expression level. Although the difference in Cav-1 expression may result from various factors such as cell type and stage of cancer (55), the tumor microenvironment and oxidative status seem to play a key role.

Several ROS have been shown to be up-regulated in the tumor microenvironment and have been implicated in the aggressive behaviors of tumor cells (5, 7). However, the mechanisms by which ROS regulate Cav-1 and tumor cell migration and invasiveness have not been thoroughly explored. In this study, we demonstrate that ROS play an important role in regulating Cav-1 expression and cell migratory functions in human lung cancer H460 cells. We also show the positive correlation between Cav-1 expression and cell motility in these cells. More importantly, we demonstrate the differential roles of individual ROS in Cav-1 expression and cell migration with $O_2^{\cdot-}$ and H_2O_2 having a negative regulatory role and $OH^{\cdot}$ playing a positive role.

The results of this study also indicate that the effect of ROS on cell migratory functions is dependent on Cav-1 expression and is associated with Akt activity. Activation of Akt by Cav-1 has been shown to mediate cancer cell migration (47–49) and is likely to play an important role in the ROS-induced effects on cell motility alterations. ROS may also regulate cell motility through other Cav-1-dependent mechanisms. For example, recent studies have shown that oxidative stress induced Cav-1 phosphorylation at tyrosine 14 (56, 57), which has been proposed to play a role in cancer cell migration through the regulation of focal adhesion (21).

The expression of Cav-1 is tightly regulated at various levels, including transcriptional and post-transcriptional levels (for review, see Ref. 51). We found that the down-regulation of Cav-1 by $O_2^{\cdot-}$ and H_2O_2 was not due to transcriptional inac-

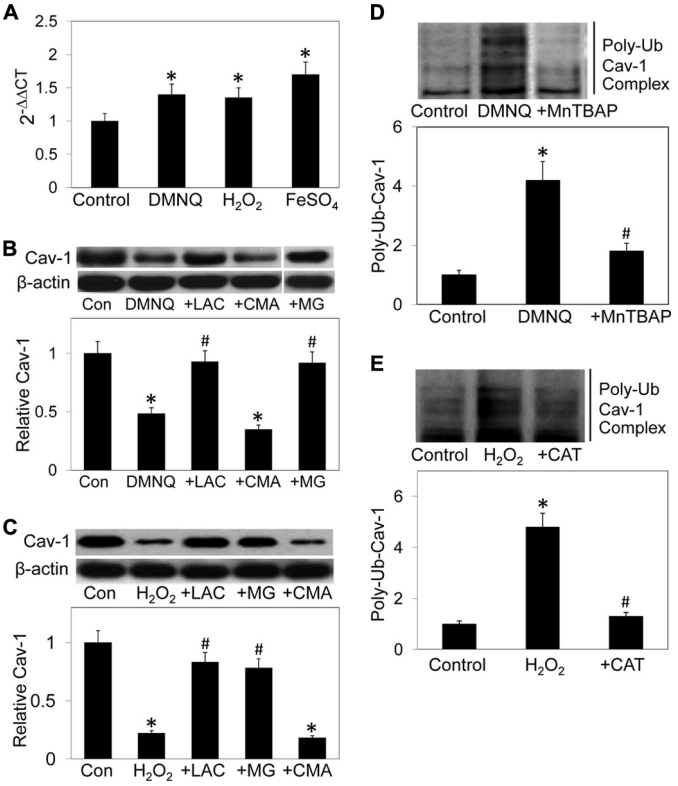

FIGURE 7. **Effects of ROS on Cav-1 mRNA expression and protein degradation.** *A*, H460 cells were either left untreated or treated with DMNQ (5 μM), H_2O_2 (100 μM), or $FeSO_4$ (50 μM) for 24 h. Cav-1 and GAPDH mRNA expressions were then determined by quantitative real time PCR. The relative mRNA expression was determined by using the comparative *CT* method as described under "Materials and Methods." *B* and *C*, H460 cells were pretreated with proteasome inhibitor lactacystin (*LAC*) (10 μM), MG132 (*MG*) (25 μM), or with lysosome inhibitor concanamycin A (*CMA*) (1 μM) for 1 h and then treated with DMNQ (5 μM) or H_2O_2 (100 μM) for 24 h. Cav-1 expression was determined by Western blots using anti-Cav-1 antibody. *Con*, control. *D* and *E*, cells were treated with DMNQ (5 μM) in the presence or absence of MnTBAP (50 μM) or with H_2O_2 (100 μM) in the presence or absence of CAT (7,500 units/ml). Cell lysates were immunoprecipitated with anti-Cav-1 antibody, and the immune complexes were analyzed for ubiquitin (*Ub*) by Western blots using anti-ubiquitin antibody. Analysis of ubiquitin was performed at 2 h post-treatment where ubiquitination was found to be maximal. Immunoblot signals were quantified by densitometry, and mean data from three independent experiments (one of which is shown here) was normalized to the result obtained in control cells. Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* nontreated control; #, $p < 0.05$ *versus* treated control.

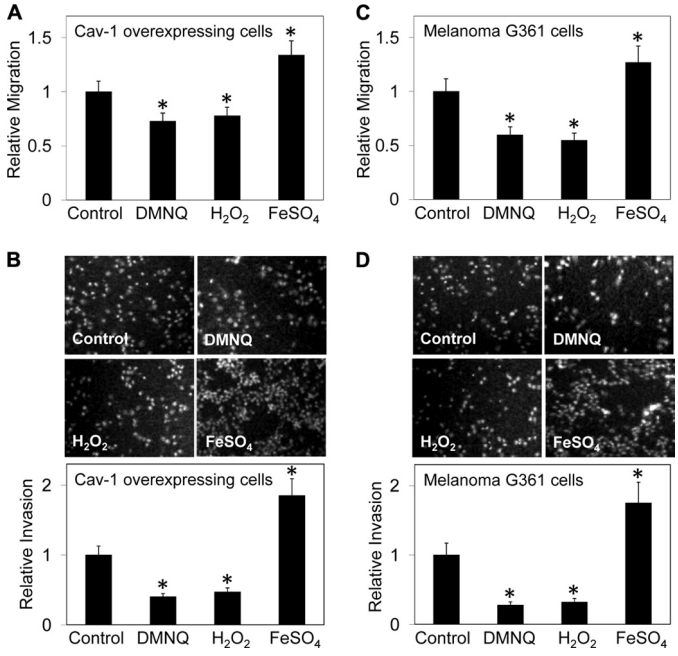

FIGURE 8. **Effects of ROS modulators on cell migration and invasion in Cav-1-overexpressing H460 cells and G361 melanoma cells.** *A* and *B*, migration and invasion of Cav-1 stably transfected H460 cells determined at 24 h after the treatment with DMNQ (5 μM), H_2O_2 (100 μM), and $FeSO_4$ (50 μM). Experiments were performed using wound assay and Transwell assay, respectively. *C* and *D*, experiments were repeated using melanoma G361 cells. Data are the means ± S.D. ($n = 3$). *, $p < 0.05$ *versus* nontreated control.

tivation because Cav-1 mRNA expression was not down-regulated but slightly increased by the $O_2^{\cdot-}$ inducer DMNQ and H_2O_2, possibly due to stress induction. Instead, such down-regulation was shown to be mediated by protein degradation via the ubiquitin-proteasome pathway. The negative regulatory roles of $O_2^{\cdot-}$ and H_2O_2 in Cav-1 expression and cell motility were confirmed by the observations that the scavengers of $O_2^{\cdot-}$ and H_2O_2 (MnTBAP and catalase) were able to reverse the effects of DMNQ and H_2O_2. These results are consistent with human clinical data showing an increase in Mn-superoxide dismutase and catalase activities in patients with non-small cell lung cancer (33, 59). Such increases may result in decreased $O_2^{\cdot-}$ and H_2O_2 levels and elevated Cav-1 and migratory activities.

The promoting role of $OH^{\cdot}$ in cancer cell migration and Cav-1 expression is unexpected and suggests the potential important role of this oxidative species in tumor progression and metastasis. To our knowledge, this is the first demonstration of the positive regulatory role of $OH^{\cdot}$ in cancer cell motility and Cav-1 expression. This finding also suggests that elevated $OH^{\cdot}$ levels may increase the risk of cancer metastasis. $OH^{\cdot}$ is a highly reactive free radical generated primarily via a biologic Fenton reaction. In addition to its known ability to induce lipid peroxidation and DNA damage, recent studies suggest that it may exert its cellular effects through the alterations of several key proteins such as p53 and metalloproteinases (58, 60). The mechanisms of $OH^{\cdot}$ regulation of cancer cell migration and invasiveness are not known and likely involve several signaling pathways. Our study provides initial evidence that this regulation is mediated, at least in part, through Cav-1 up-regulation.

In conclusion, we demonstrate that Cav-1 plays an important role as a positive regulator of cancer migration and invasion in human lung carcinoma H460 cells. Our results also reveal the differential roles of individual ROS in cancer cell motility and Cav-1 expression, which could be important in understanding tumor progression and metastasis. Our results also provide evidence supporting the role of $OH^{\cdot}$ as a key determinant and positive regulator of cancer cell invasiveness. This new finding suggests a linkage between elevated $OH^{\cdot}$ formation and increased risk of tumor formation and metastasis.

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