

Reduction in MnSOD promotes the migration and invasion of squamous carcinoma cells

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Abstract. Reactive oxygen species (ROS) homeostasis is maintained at a higher level in cancer cells, which promotes tumorigenesis. Oxidative stress induced by anticancer drugs may further increase ROS to promote apoptosis, but can also enhance the metastasis of cancer cells. The effects of ROS homeostasis on cancer cells remain to be fully elucidated. In the present study, the effect of a reduction in manganese superoxide dismutase (MnSOD) on the migration and invasion of A431 cells was investigated. Our previous micro-assay data revealed that the mRNA expression of MnSOD was higher in the invasive A431-III cell line compared with that in the parental A431 cell line (A431-P). In the present study, high protein levels of MnSOD and H₂O₂ production were observed in A431-III cells; however, catalase protein levels were significantly lower in A431-III cells compared with those in the A431-P cell line. The knockdown of MnSOD increased H₂O₂ levels, enzyme activity, the mRNA levels of matrix metalloproteinase-1, -2 and -9, and the migratory and invasive abilities of the cells. Inducing a reduction in H₂O₂ using diphenyleneiodonium (DPI) and N-acetyl-L-cysteine decreased the migratory abilities of the cell lines, and DPI attenuated the migratory ability that had been increased by MnSOD small interfering RNA knockdown.

Luteolin (Lu) and quercetin (Qu) increased the expression of catalase and reduced H₂O₂ levels, but without an observed change in the protein levels of MnSOD. Taken together, these data suggest that reduced MnSOD may induce ROS imbalance in cells and promote the metastatic ability of cancer cells. Lu and Qu may attenuate these processes and may be promising potential anticancer agents.

Introduction

The overall redox environment in the cytosol and mitochondria is defined by the balance of reactive oxygen species (ROS). Elevated ROS production by mitochondria increases ROS-scavenging activity in order to maintain homeostasis (1). It is well established that high levels of ROS increase cellular oxidative stress, which leads to protein, lipid and DNA oxidation, and promotes tumor development. An increase in antioxidant ability sensitizes malignant cancer cells to chemotherapy. Therefore, the ROS balance in mitochondria serves an important role in tumor cells (2).

Manganese superoxide dismutase (MnSOD) regulates ROS homeostasis and exhibits tumor-suppressive and cancer-promoting functions (3). The expression of MnSOD has been reported to be reduced in cancer cells compared with that in normal tissues. The forced overexpression of MnSOD has resulted in delayed tumor growth in several cancer xenograft studies in mice (4,5). A reduced expression of MnSOD has been reported in early tumor lesions, with the upregulation of MnSOD observed in metastatic tumor cells. In fibrosarcoma cells, the overexpression of MnSOD has been demonstrated to enhance metastatic capacity by modulating the expression of matrix metalloproteinase (MMP)-1 and other MMP family members (6); the pro-metastasis function of MnSOD was previously demonstrated to be hydrogen peroxide (H₂O₂)-dependent (7). In lung adenocarcinoma, MnSOD upregulates forkhead box protein M1/MMP-2 to promote tumor invasion (8). The expression of MnSOD has

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also been found to be upregulated in patients with primary gastric cancer and lymph node metastases (9). Additionally, the overall survival rate and pathologic tumor stages of patients with nasopharyngeal carcinoma was reported to be negatively correlated with the expression of MnSOD (10). The upregulation of MnSOD in cancer cells may increase the production of H_2O_2 to promote tumor growth in later stages of prostate, colon and lung cancer. ROS homeostasis is altered by the superoxide/ H_2O_2 ratio, which can promote the proliferation and metastasis of cancer cells (11), and the scavenging of mitochondria superoxide may reduce the metastatic ability of cancer cells (12,13).

Flavonoids are plant phenolic compounds with antioxidant and chelating activities (14-18). Numerous studies have indicated that two dietary flavonoid constituents, luteolin (Lu), a flavone, and quercetin (Qu), a flavonol, generally appear to be the most potent among plant flavonoids in terms of their *in vitro* biological activities (19-21). These flavonoids exhibit a variety of anticancer effects, including inhibition of cell growth and kinase activity, induction of apoptosis, stimulation of differentiation, suppression of MMP secretion, tumor cell adhesion, invasive behavior, metastasis and angiogenesis (21,22). Lu has been reported as a potent anticancer agent in squamous cell carcinoma cells and other cancer cell lines (23-26). Lu has also been reported to alter the activity of antioxidant enzymes in cancer cells. In CH27 cells, Lu induced apoptosis and increased the activation and expression of copper-dependent superoxide dismutase (CuSOD) and catalase (27), and has been observed to decrease the cisplatin-induced renal production of ROS by increasing the expression of CuSOD and catalase (28). Qu has also been reported to induce catalase activity in studies investigating ROS; catalase activity was reduced in a 3-nitropropionic acid-induced mice model of Huntington's disease, whereas treatment with Qu reversed the reduced catalase activity in the model (29). In a toxicology study, the co-administration of Qu with chromium led to significantly enhanced expression of catalase in mice compared with that in mice administered with chromium alone (30).

Our previous study established the invasive A431-III cell line from the parental A431 (A431-P) cell line (31). The invasive A431-III cells expressed higher levels of MMP-2 and -9 compared with levels in the A431-P cell line, and exhibited high metastatic ability mediated via epithelial-mesenchymal transition (EMT) signaling coordinated by Snail (32). Additionally, our previous study indicated that transglutaminase 2 contributes to the metastasis of A431-III cells by activating phosphatidylinositol-3-kinase (PI3K) and nuclear factor- κ B signaling, which induces the expression of Snail and MMP-9 (33). The flavonoids Lu and Qu have been shown to inhibit EMT signaling in squamous cell carcinoma cells (34). Additionally, protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/c-Myc signaling induced the expression of 40S ribosomal protein S (RPS)12 and RPS19 in A431-III cells and promoted metastasis, which was attenuated by Lu and Qu (35,36). Furthermore, Lu and Qu reduced the expression of UBE2S to attenuate the activation of hypoxic and EMT signaling in cancer cells (37). Taken together, these previous findings suggest that Lu and Qu may be promising candidates as anticancer agents (18).

The present study aimed to investigate the effects of an ROS imbalance, via the knockdown of MnSOD and the use of antioxidant reagents, on the migratory and invasive abilities of A431-P and A431-III cancer cells. The effects of Lu and Qu on the production of H_2O_2 and expression of oxidative enzymes were also analyzed.

Materials and methods

Materials. A431-P (A431) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). A431-III cells were generated in our laboratory (Ming-Ting Lee, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan) from the parental A431-P tumor cells (31). RPMI-1640 and fetal bovine serum (FBS) were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-MnSOD and anti- β -actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-Cu/zinc (Zn)SOD antibody was obtained from Merck KGaA (Darmstadt, Germany). Anti-catalase antibody was obtained from Chemicon International (Thermo Fisher Scientific, Inc.). Polymerase chain reaction (PCR) forward and reverse primers were purchased from Purigo Biotech (Taipei, Taiwan). Luteolin (purity $\geq 95\%$) was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Quercetin (purity $\geq 95\%$) was purchased from Nacalai Tesque (Kyoto, Japan). Agarose and dimethyl sulfoxide (DMSO) were purchased from Merck KGaA. Epidermal growth factor was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA) and dissolved in RPMI-1640 medium. Unless otherwise indicated, all other reagents were obtained from Merck KGaA. Lu and Qu were dissolved in 100% DMSO, and their concentrations were adjusted to obtain 100 mM stock solutions.

Cell culture. The A431-P and A431-III cells were cultured as described in our previous report (31). The A431-P and A431-III cells were incubated in a 5% CO_2 air atmosphere at 37°C with RPMI-1640 medium containing 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.).

Preparation of cell lysates. All procedures for preparing cell lysates were performed on ice. The A431-P and A431-III sub-lines were harvested and washed three times with cold phosphate-buffered saline (PBS; Bioman, Taipei, Taiwan) and then were lysed in gold lysis buffer [20 mM Tris-HCl, (pH 7.9), 1 mM EGTA, 0.8% NaCl, 0.1 mM, β -glycerylphosphate, 1 mM sodium pyrophosphate, 10 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4 , 10% glycerol and 1% Triton X-100] containing 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin.

Transfection of small interfering RNA (siRNA). MnSOD siRNA and non-specific RNA (both from Ambion; Thermo Fisher Scientific, Inc.) were dissolved in nuclease-free water to a concentration of 10 μ M. The A431-P and A431-III cells (5×10^5) were plated onto 6-well plates and allowed to adhere overnight. 2 μ l Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.; 2 μ l per well) was added into 100 μ l serum-free RPMI-1640, thoroughly mixed and incubated at room temperature for 5 min. MnSOD siRNA (4 μ l per well) was

added into 100 μ l of serum-free RPMI-1640, thoroughly mixed and then combined with the diluted Lipofectamine 2000. The siRNA/Lipofectamine[®] mixture was gently mixed and incubated at room temperature for 30 min, and then added into the 6-well plate containing cells in 2 ml serum-free RPMI-1640 for 24 h. All subsequent assays were performed at 24 h post-transfection.

Wound-healing assay. The A431-P (1×10^6 cells/well) and A431-III cells (1×10^6 cells/well) were seeded into 6-well plates and incubated overnight. Manual scratching using a pipette tip and subsequent washing with PBS was performed to create the wound in the cell monolayers. The A431-P cells were treated with 10, 30 and 50 μ M of H₂O₂, and the A431-III cells were treated with 1.5, 2.5 and 5 μ M of N-acetyl-L-cysteine (NAC; Sigma-Aldrich; Merck KGaA) at 37°C for 16-h. Additionally, A431-P and A431-III cells transfected with MnSOD siRNA was pre-treated with 20 μ M of diphenyleneiodonium (DPI; Sigma-Aldrich; Merck KGaA) at 37°C for 3-h and then cultured for an additional 17 h at 37°C were also analyzed. Images of the monolayers were captured following wounding using phase-contrast microscopy and an Olympus IX70 camera (Olympus Corporation, Tokyo, Japan) to determine the size of the wound.

Migration assay. Transwell chambers with polycarbonate filters were coated with extracellular matrix (both from BD Biosciences, Franklin Lakes, NJ, USA) for 1 h at 25°C. Culture medium was added to the lower compartment of the chamber. The upper chambers were loaded with 10^5 cells/well in serum-free medium and the lower chambers were filled with medium containing 10% FBS as a chemoattractant for the cells. The cells were incubated at 37°C in a humidified atmosphere (95% air and 5% CO₂) for 10 h. Following incubation, the invading cells were fixed with 1% glutaraldehyde/PBS for 15 min at 25°C, and then stained with crystal violet for 30 min. Cells on the upper layer of the filter were removed, and images were captured using an Olympus SZ-PT (Olympus Corporation) microscope and counted. Data of the migrated cell numbers represent the average of three typical fields per sample.

Invasion assay. Chambers (Corning, Inc., Corning, NY, USA) with an 8- μ m pore size were pre-coated with 20 μ g/100 μ l EHS Matrigel (BD Biosciences). Following drying, the chambers were rinsed with 100 μ l serum-free RPMI medium for rehydration. The cells (10^5 cells/well) in serum-free medium were loaded into the chamber and inserted into a 24-well plate (Corning, Inc.). A 0.5-ml aliquot of medium supplemented with 10% FBS was added to the lower wells to serve as a chemoattractant for the cells. The Matrigel invasion chambers were incubated at 37°C for 60 h in 0.5% CO₂. Following incubation, the invading cells were fixed with 1% glutaraldehyde/PBS for 15 min and then stained with crystal violet for 30 min. Images of the cells on the upper layer of the chamber were captured with an Olympus SZ-PT microscope and counted.

Crystal violet staining. Following removal of the cell culture medium, the cells were washed twice with PBS and then fixed using 1% glutaraldehyde for 15 min. The glutaraldehyde was

removed, and the cells were washed twice with PBS, following which crystal violet (Koch-Light Research Laboratories, Ltd., Gauteng, South Africa) stain was added for 30 min. The cells were then washed twice with distilled water. The optical density of the stained cells was measured at 595 nm.

Western blot analysis. The protein concentration was determined by Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). The 20 μ g of total protein was used to analyzed and separated by 10% SDS PAGE, and then transferred onto a nitrocellulose membrane (Pall Life Sciences, Port Washington, NY, USA). The nitrocellulose membrane was blocked with 3% bovine serum albumin at room temperature for 1 h and then incubated with the anti-CuSOD (1:5,000; cat. no. MABC684; Merck KGaA), anti-MnSOD (1:5,000; cat. no. sc-137254; Santa Cruz Biotechnology, Inc.), anti-catalase (1:1,000; cat. no. AML0010; Thermo Fisher Scientific, Inc.) and anti- β -actin (1:5,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) antibodies in blocking solution at 4°C overnight. The membranes were thoroughly washed four times with TBST [20 mM Tris-HCl (pH 7.6), 0.8% NaCl, and 0.25% Tween-20] and twice with Tris-buffered saline, and were then incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. 715-035-150; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in TBST for 1 h at room temperature. Following four washes with TBST, the blots were stained using the chemiluminescent SuperSignal substrate (Thermo Fisher Scientific, Inc.) and immunoreactive signals were visualized by exposure to X-ray film.

SOD activity gel assay. SOD activity was measured using the modified nitroblue tetrazolium (NBT) method (38). The 20 μ g of total protein from each sample were separated using a native 10% polyacrylamide gel. Following three washes with 2% Triton X-100 and distilled water, the gel was incubated with 2.43 mM NBT (Sigma-Aldrich; Merck KGaA) in the dark for 20 min at 25°C, then 28 μ M riboflavin and 28 mM TEMED (Sigma-Aldrich; Merck KGaA) were added to react for 15 min in the dark at 25°C. Following exposure of the gel to light, the achromatic bands corresponding to SOD activity appeared on a dark-purple background.

Gelatin zymography. The activity of secreted MMP was analyzed by casein and gelatin zymography (39). The A431-P and A431-III sub-lines (5×10^5 cells/well) were seeded into 6-well plates and then cultured in 1.5 ml of serum-free medium for 24 h. The conditioned medium was collected and separated on a gradient polyacrylamide gel that contained gelatin or casein as a substrate. The gel was incubated with substrate buffer [50 mM Tris-HCl (pH 8.0), 5 mM CaCl₂ and 0.02% NaN₃] at 37°C for 48 or 96 h prior to staining with Coomassie blue (0.25% R250, 50% ethanol and 10% acetic acid). The gel was destained with destaining buffer (20% ethanol and 10% acetic acid) until the achromatic bands appeared on a blue background.

ROS measurement. The production of H₂O₂ was measured using an Amplex[®] Red Hydrogen Peroxide/Peroxidase Assay kit (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Briefly, the A431-P and A431-III sub-lines

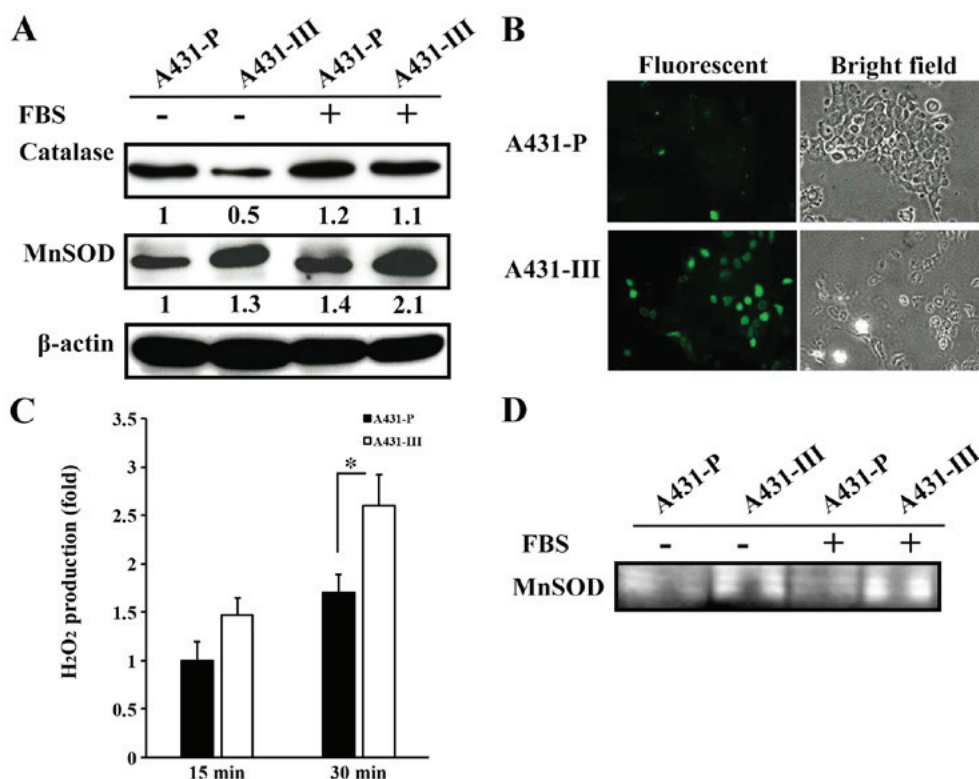


Figure 1. H_2O_2 production is regulated by MnSOD and catalase in A431-P and A431-III cells. (A) Protein levels of MnSOD and catalase in A431-P and A431-III cells cultured with or without FBS were determined by western blot analysis. (B) Production of H_2O_2 in A431-P and A431-III cells was analyzed using 2',7'-dichlorofluorescein diacetate incubation followed by fluorescent microscopy (magnification, x200) and (C) detection of the fluorescent intensity using the Amplex[®] Red Hydrogen Peroxide/Peroxidase Assay kit. Statistical significance was determined by a one-way ANOVA with Tukey's test (* $P < 0.05$). (D) Enzyme activity of MnSOD was analyzed using the nitroblue tetrazolium method. H_2O_2 , hydrogen peroxide; MnSOD, manganese superoxide dismutase; FBS, fetal bovine serum.

(1×10^4 cells/well) were seeded into a 96-well plate (Corning, Inc.) and incubated overnight. The A431-III cells were treated with 20 μ M of Lu and Qu at 37°C for 24-h, following which the cells were analyzed using Amplex Red reagent (Thermo Fisher Scientific, Inc.) and a SPARK[®] plate reader (Tecan Group, Inc., Männedorf, Switzerland) to measure the absorbance. For fluorescent microscopy, 2',7'-dichlorofluorescein diacetate (2',7'-DCF-DA; Thermo Fisher Scientific, Inc.) was used as the probe to detect ROS, followed by observation under an Olympus IX70 (Olympus Corporation) microscope.

Reverse transcription-polymerase chain reaction (RT-PCR) messenger (m)RNA analysis. The cells were cultured in 10-cm dishes to 90% confluence, detached using 0.25% trypsin for 10 min and then blocked in medium containing 10% FBS medium. Total RNA was extracted by using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. The SuperScript III First-Strand Synthesis system (Thermo Fisher Scientific, Inc.) was used for first-strand cDNA synthesis according to the manufacturer's protocol. For PCR amplification, primers were designed according to DNA sequences as follows: MMP-1, forward, 5'-GGA GGA AAT CTT GCT CAT-3' and reverse, 5'-CTC AGA AAG AGC ATC-3'; MMP-3, forward, 5'-ATT ATA CAC CAG ATT TGC CAA AAG A-3' and reverse, 5'-AAA AGA ACC CAA ATT CTT CAA AAA C-3'; MMP-9, forward, 5'-CCG CGA CAC CAA ACT GGA T-3' and reverse, 5'-TGT ACC GCT ATG GTT ACA C-3'; and β -actin, forward, 5'-GCT CGT CGT CGA CAA CGG CTC-3' and reverse, 5'-CAA ACA TGA TCT GGG TCA

TCT TCTC-3'. Amplification of β -actin cDNA was used as the control reference gene. The following profile was used for PCR amplification of 500 ng of cDNA by KAPA HiFi PCR Kits (Kapa Biosystems, Woburn, MA, USA) in a GeneAmp PCR System 9700 (Thermo Fisher Scientific, Inc.): an initial denaturation step at 96°C for 5 min, followed by 30 cycles of 96°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min.

Statistical analysis. Data from three independent experiments are expressed as the mean \pm standard deviation. For comparisons between two groups, statistical analysis was determined using Student's unpaired t-test (SPSS Statistics ver. 24; IBM, Chicago, IL, USA). For comparison of more than two groups, one-way analysis of variance followed by Tukey's test was used (SPSS Statistics ver. 24; IBM). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Levels of MnSOD, catalase, and H_2O_2 in the A431-III sub-line. Our previous studies identified the mechanism of metastatic activation in an A431-III cell model (32-36). ROS signaling is reported to activate metastasis in cancer cells (2,40). To analyze the role of MnSOD in the metastasis of cancer cells, the expression levels of MnSOD and catalase were compared in A431-III and A431-P cells. The protein levels of MnSOD were higher in the A431-III cells than in the A431-P cells cultured with or without FBS; however,

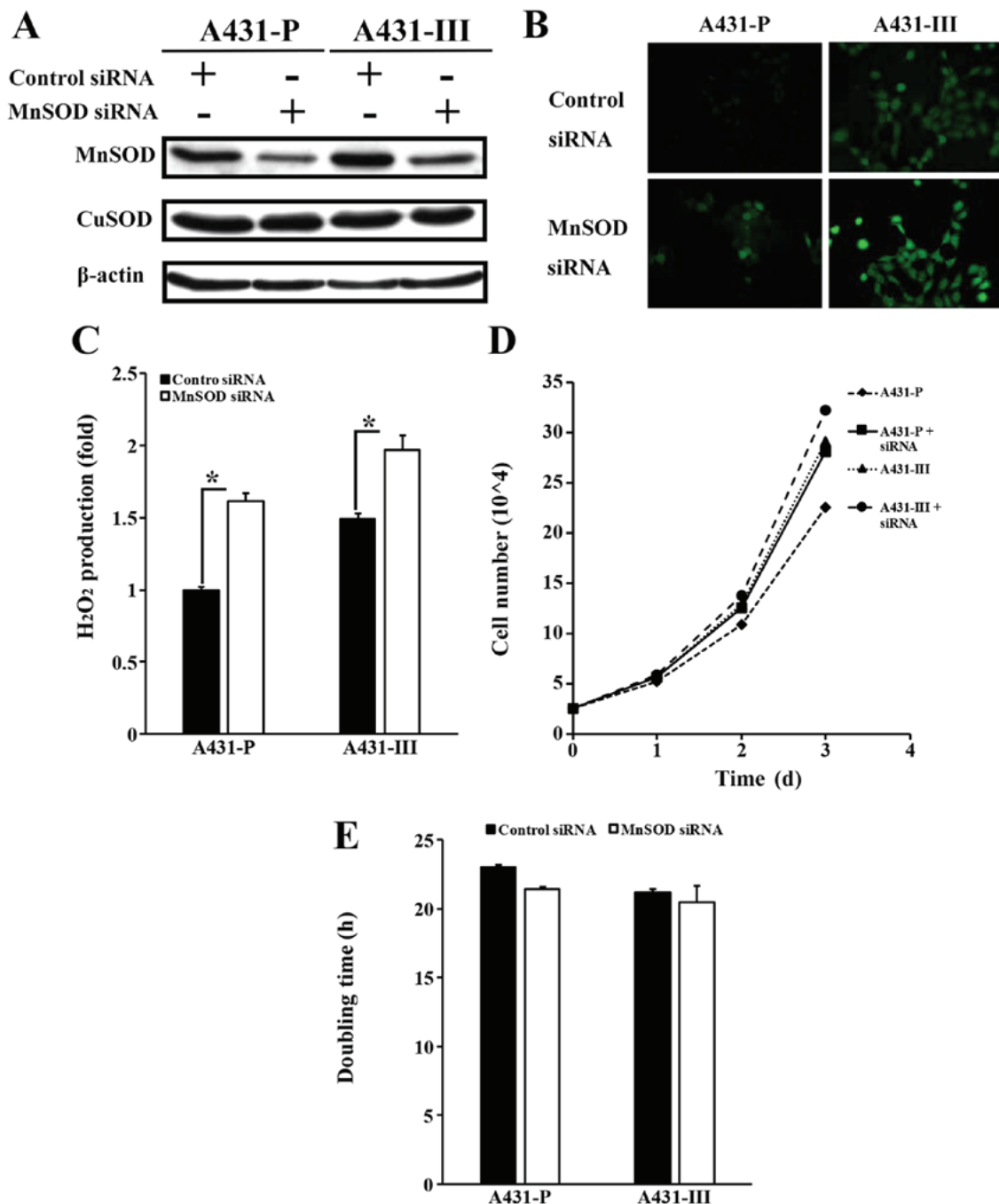


Figure 2. MnSOD knockdown increases the production of H₂O₂. (A) Protein levels of MnSOD and CuSOD were determined using western blot analysis following transfection with MnSOD siRNA and control siRNA in A431-P and A431-III cells, respectively. (B) Production of H₂O₂ in A431-P and A431-III cells was measured using 2',7'-dichlorofluorescein diacetate incubation followed by fluorescent microscopy (magnification, x200) and (C) detection of the fluorescent intensity using the Amplex[®] Red Hydrogen Peroxide/Peroxidase Assay kit. (D) Cell growth and (E) doubling times of A431-P and A431-III cells transfected with MnSOD and control siRNA. MnSOD, manganese superoxide dismutase; H₂O₂, hydrogen peroxide; CuSOD, copper superoxide dismutase; siRNA, small interfering RNA. Statistical significance was determined by a one-way ANOVA with Tukey's test (*P<0.05).

catalase levels were lower in the A431-III cells than in the A431-P cells (Fig. 1A). Analysis using the oxidant-sensitive dye 2',7'-DCF-DA revealed that H₂O₂ production 15 and 30 min following dye application was increased 1.7- and 2.6-fold, respectively, in the A431-III cells compared with that in the A431-P cells (Fig. 1B and C). The enzymatic activity of MnSOD was also higher in the A431-III cells than in the A431-P cells (Fig. 1D). These results suggest that the higher production of H₂O₂ in A431-III cells may be regulated by MnSOD and catalase.

Knockdown of the expression of MnSOD enhances the production of H₂O₂. To analyze the effect on the production of H₂O₂, siRNA knockdown was used to reduce the expression of MnSOD. Western blotting confirmed that the protein level of MnSOD was reduced by the siRNA in the A431-P and A431-III cell lines. The protein level of CuSOD was not altered by the siRNA (Fig. 2A). The knockdown of MnSOD increased the production of H₂O₂ by 1.6- and 2.0-fold in the A431-P and A431-III cell lines, respectively, compared with that in the control groups (Fig. 2B and C). The cell numbers and doubling

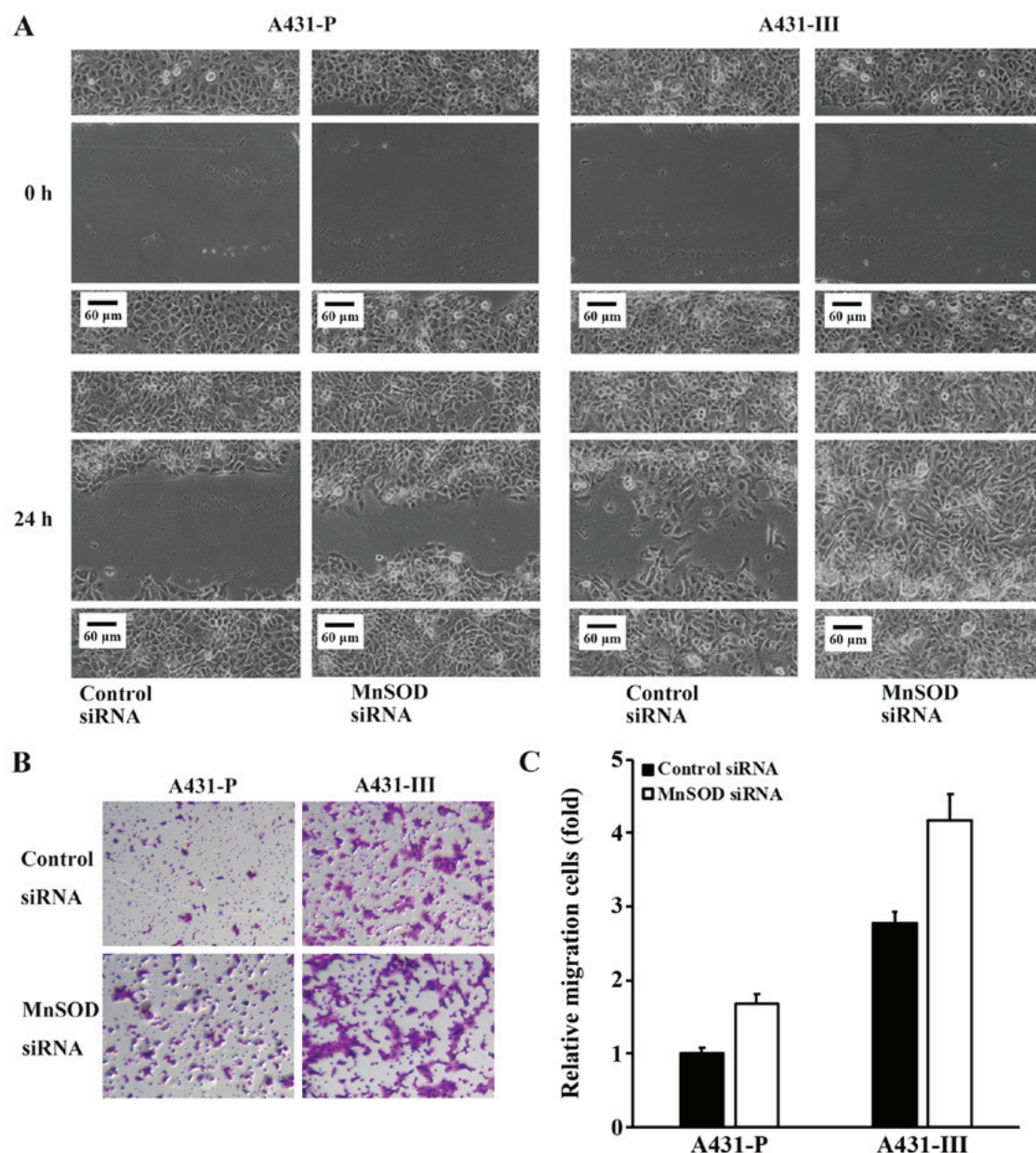


Figure 3. MnSOD knockdown increases the migratory ability of A431-P and A431-III cells. (A) Wound-healing assay and (B) migration assay using A431-P and A431-III cells transfected with MnSOD siRNA and control siRNA (magnification, x200). (C) Quantification of migration ability. MnSOD, manganese superoxide dismutase; siRNA, small interfering RNA.

times of the A431-P and A431-III cells were not significantly affected by the knockdown of MnSOD (Fig. 2D and E). These results suggest that loss of the expression of MnSOD may increase the production of H_2O_2 .

Knockdown of the expression of MnSOD increases the migratory ability of cancer cells. To analyze the effect of altering the expression of MnSOD on cell migratory ability, wound-healing and cell-migration assays were performed using the A431-P and A431-III cells. The knockdown of MnSOD increased the migratory ability of the A431-P and A431-III cells in the wound-healing experiment (Fig. 3A); migration was increased 1.68-fold in the A431-P cells with MnSOD siRNA knockdown, compared with that of the cells transfected with control siRNA. The migration ability of the A431-III cells increased from 2.77-fold to 4.17-fold by MnSOD siRNA knockdown compared

with that of cells in the control siRNA group (Fig. 3B and C). These results suggest that the reduced expression of MnSOD may increase the migratory ability of cancer cells.

Knockdown of the expression of MnSOD increases the invasive ability of cancer cells and activates MMPs. To further analyze the role of MnSOD in the invasive ability of cancer cells, an invasion assay was performed using A431-P and A431-III cell lines transfected with MnSOD siRNA. The knockdown of MnSOD in A431-P cells increased the number of invasive cells by 1.9-fold compared with that of the control siRNA-transfected cells. The invasive ability of the A431-III cells was increased by MnSOD knockdown from 2.58-fold in the control group to 3.6-fold (Fig. 4A and B). The invasive ability of the cancer cells is associated with the activity of MMPs. In further analysis of metalloproteinases activity,

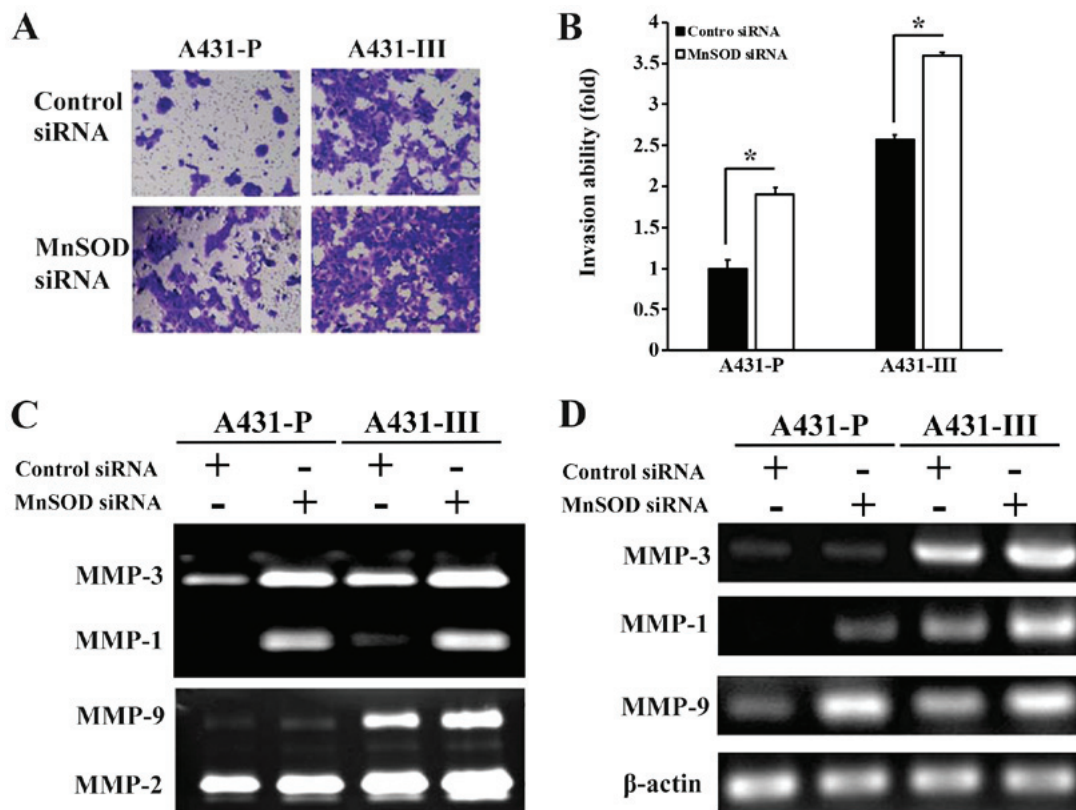


Figure 4. MnSOD knockdown increases the invasive abilities of A431-P and A431-III cells and increases the activity of metalloproteinases. (A) Invasion abilities of A431-P and A431-III cells were examined following transfection with MnSOD siRNA and control siRNA (magnification, x200). (B) Cells that penetrated through the Matrigel to the lower surface of the filters were stained and counted by microscopy. Statistical significance was determined by a one-way ANOVA with Tukey's test (* $P < 0.05$). (C) Conditioned media of A431-P and A431-III cells transfected with MnSOD siRNA and control siRNA were collected and normalized by cell numbers. Casein and gelatin zymographic assays were used to analyze the enzyme activities of MMP-1, -2, -3 and -9. (D) Reverse transcription-polymerase chain reaction was used to analyze the mRNA levels of MMP-1, -3 and -9 following MnSOD siRNA and control siRNA transfection. MnSOD, manganese superoxide dismutase; MMP, matrix metalloproteinase.

the protein activity (Fig. 4C) and mRNA (Fig. 4D) levels of MMP-1, -3 and -9 were increased by MnSOD knockdown. Collectively, these results suggest that the invasive ability of cancer cells was increased by MnSOD siRNA knockdown, which also increased the expression and enzymatic activities of MMP-1, -3 and -9.

Migratory ability of cancer cells is regulated by H_2O_2 . In the present study, the knockdown of MnSOD increased the production of H_2O_2 , and increased the migratory and invasive abilities of cancer cells. To elucidate the roles of H_2O_2 in the migratory ability of cancer cells, the oxidant-sensitive dye 2',7'-DCF-DA and wound-healing experiments were performed using A431-III cells. The production of H_2O_2 was reduced by treatment with 2.5 μ M of DPI and 5 mM of NAC (Fig. 5A). To further analyze the effect on the migratory ability of cancer cells by H_2O_2 production, wound-healing experiments were performed. The migratory ability of A431-P cells was increased following treatment with H_2O_2 in a dose-dependent manner (Fig. 5B); however, the migratory ability of A431-III cells was reduced by DPI (1.5 and 2.5 μ M) and NAC (5 μ M; Fig. 5C). These data suggest that the migratory ability of cancer cells is regulated by the levels of H_2O_2 .

DPI reverses the migratory ability induced by MnSOD knockdown. MnSOD siRNA was used to elucidate the role of

H_2O_2 in the enhanced migratory ability of cancer cells induced by MnSOD knockdown. MnSOD siRNA increased the production of H_2O_2 by 1.46-fold in the A431-P cells compared with cells in the control siRNA group. In the A431-III cells, the production of H_2O_2 was increased from 1.3-fold in the control group to 2.1-fold in the MnSOD knockdown cells. The production of H_2O_2 increased by MnSOD siRNA was reduced to 0.1- and 0.21-fold by 2.5 μ M of DPI treatment in the A431-P and A431-III cells, respectively (Fig. 6A). The migratory abilities of the A431-P and A431-III cells were increased by MnSOD siRNA, and this effect was abrogated by treatment with 2.5 μ M of DPI (Fig. 6B). These results suggest that altered MnSOD levels/activity may increase the production of H_2O_2 , resulting in an increase in the migratory ability of cancer cells.

Lu and Qu reduce the production of H_2O_2 and increase the expression of catalase. In our previous reports, Lu and Qu were demonstrated to inhibit the metastasis of cancer cells (34-37). In the present study, the migratory and invasive abilities of cancer cells were associated with the level of H_2O_2 . To analyze the effects of Lu and Qu on the production of H_2O_2 , the cells were probed with the oxidant-sensitive dye, 2',7'-DCF-DA, following treatment of the A431-III cells with Lu and Qu. The H_2O_2 level was significantly reduced, by 0.6- and 0.5-fold, following treatment with 20 μ M of Lu and Qu, respectively, compared with that in the control group (Fig. 7A and B). In

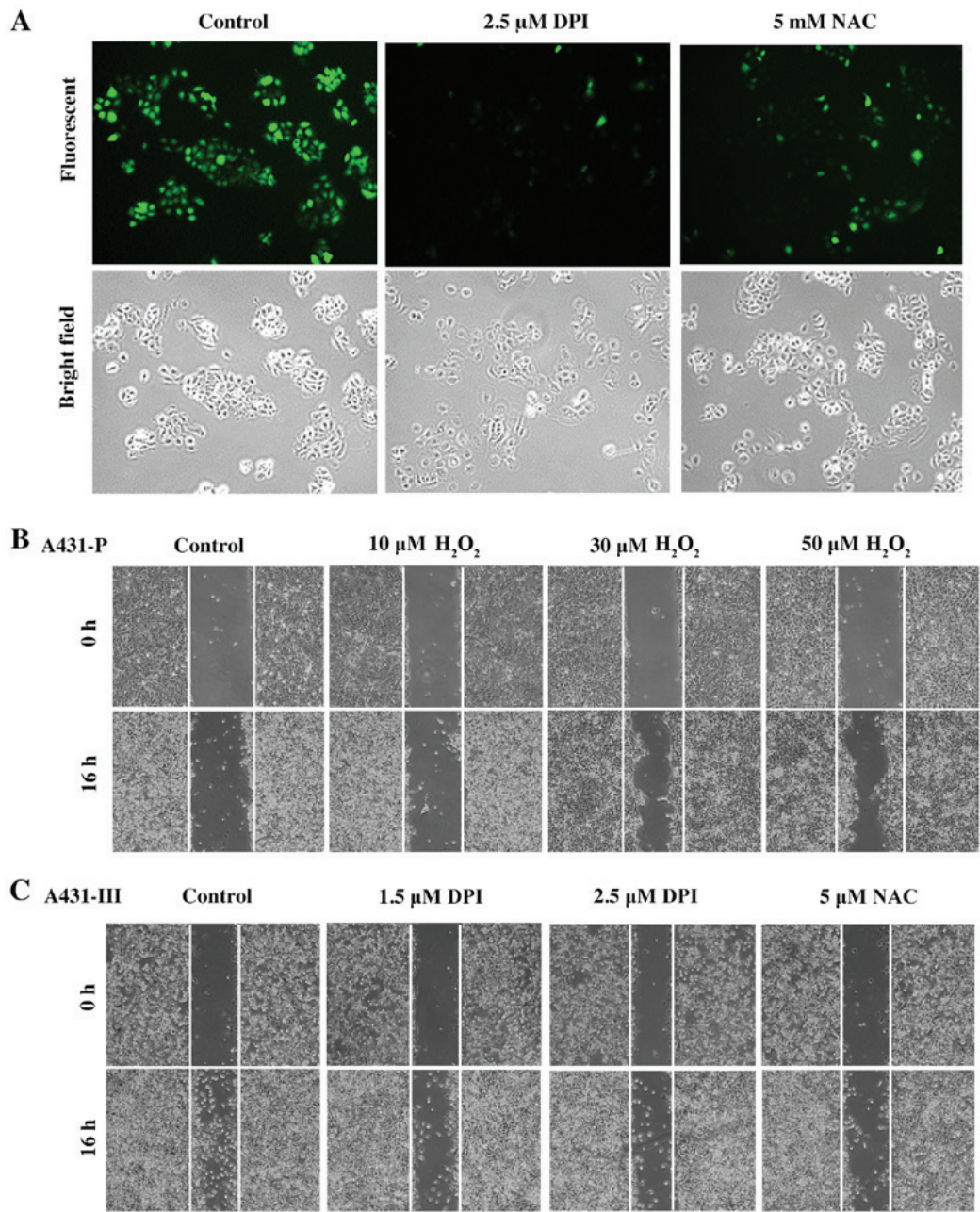


Figure 5. H_2O_2 promotes the migratory abilities of A431-P and A431-III cells. (A) H_2O_2 in A431-P and A431-III cells following treatment with 2.5 μM of DPI and 5 mM of NAC was measured using 2',7'-dichlorofluorescein diacetate and fluorescent microscopy (magnification, x200). (B) A wound-healing assay was used to analyze the migratory ability in A431-P cells following treatment with 10, 30 and 50 μM of H_2O_2 for 16 h (magnification, x100). (C) A wound-healing assay was used to analyze the migratory ability of A431-III cells following treatment with 1.5 and 2.5 μM of DPI and 5 μM of NAC for 16 h (magnification, x100). H_2O_2 , hydrogen peroxide; DPI, diphenyleneiodonium; NAC, N-acetyl-L-cysteine.

further analysis of the effects of Lu and Qu on the expressions of MnSOD and catalase, the protein levels of MnSOD did not alter, however, the levels of catalase were increased (Fig. 7C). These data suggest that Lu and Qu reduced H_2O_2 levels by increasing the protein level of catalase, without an effect on the level of MnSOD.

Discussion

Cancer cells exhibit high ROS levels and increased antioxidant capacity (2). In A431-III cells, the high expression of MnSOD and low expression of catalase may increase the antioxidant capacity of cancer cells. The knockdown of MnSOD in

A431-III cells may induce an imbalance of ROS and increase the migratory and invasive ability of cancer cells. In the present study, Lu and Qu reduced the production of H_2O_2 and increased the expression of catalase (Fig. 7).

ROS are reported to downregulate catalase via the activation of PI3K/Akt signaling to reduce the activity of forkhead box protein O1 (FoxO1) in mesangial cells (41). In our previous reports, Lu and Qu inhibited Akt/mTOR/cMyc signaling to repress RPS19- and RPS12-activated metastasis in A431-III cells (35,36). In the present study, the levels of H_2O_2 were higher and catalase levels were lower in A431-III cells than in A431-P cells; the low of catalase in A431-III cells may be caused by the H_2O_2 -induced activation of Akt/FoxO1

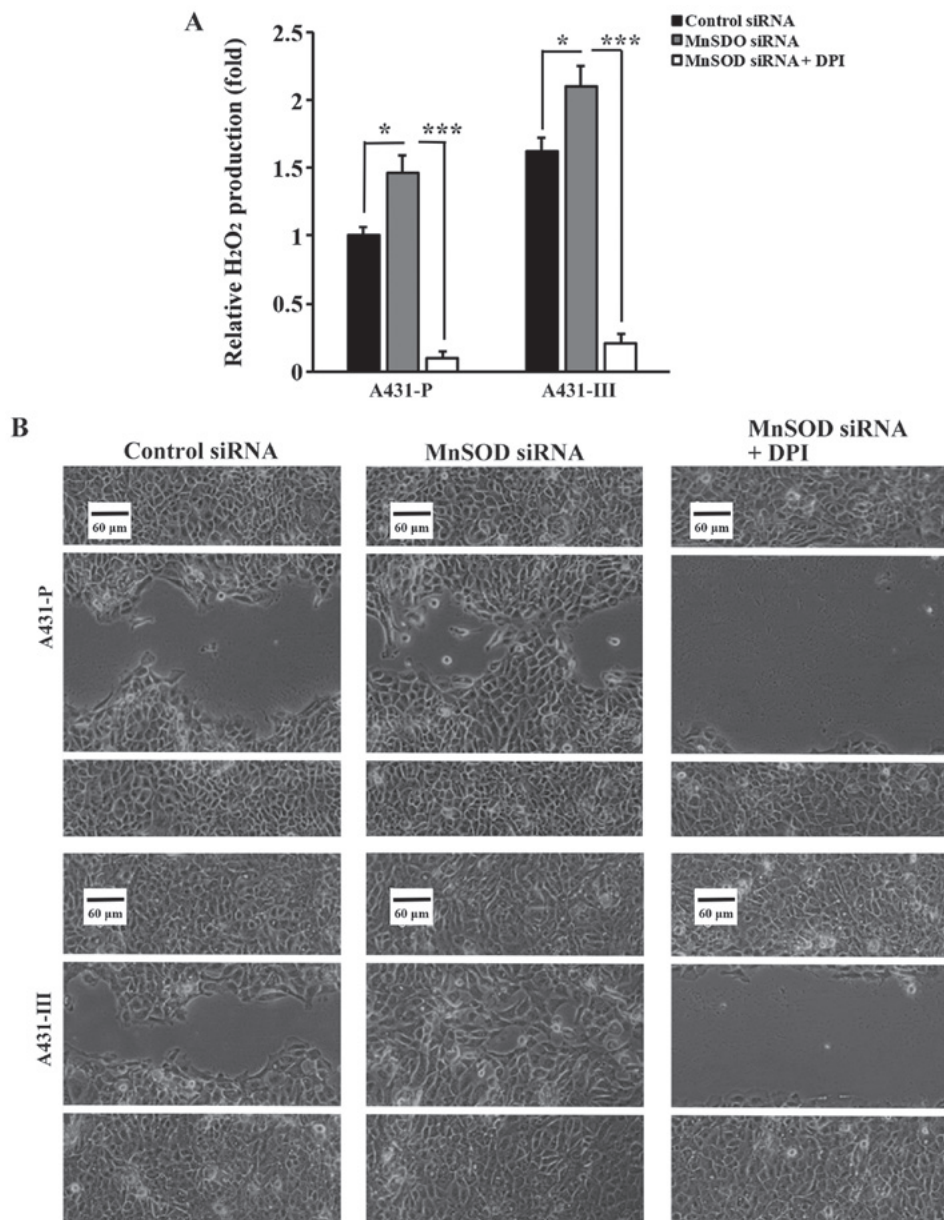


Figure 6. DPI reverses H₂O₂ production and the migratory ability induced by MnSOD knockdown in A431-P and A431-III cells. Following transfection of A431-P and A431-III cells with MnSOD siRNA and control siRNA, cells were treated with or without DPI. (A) Production of H₂O₂ was measured by 2',7'-dichlorofluorescein diacetate and fluorescence incubation, and the fluorescent intensity was detected using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay kit. Statistical significance was determined by a one-way ANOVA with Tukey's test (*P<0.05, ***P<0.001). (B) A wound-healing assay was performed to analyze the migratory abilities of A431-P and A431-III cells transfected with control siRNA, and MnSOD siRNA alone or combined with DPI (magnification, x200). MnSOD, manganese superoxide dismutase; DPI, diphenyleneiodonium; H₂O₂, hydrogen peroxide; siRNA, small interfering RNA.

signaling. Lu and Qu increased the expression of catalase and reduced the production of H₂O₂ in A431-III cells. The inhibition of Akt signaling by Lu and Qu may activate FoxO1 and, thus increase the expression of catalase (41,42). Further analysis is required to elucidate the mechanism by which Lu and Qu increase the expression of catalase.

The knockdown of MnSOD using siRNA reduces the migratory and invasive abilities of cancer cells (43,44); however, in a previous study, the reduced migratory and invasive abilities caused by MnSOD knockdown were restored by the combined knockdown of homeobox protein Nkx2.1 in lung adenocarcinomas (44). Reports have demonstrated that the migratory and invasive abilities of cancer cells are regulated by the expression of MnSOD and other factors in cancer

cells (7,8,43). In the present study, the knockdown of MnSOD by siRNA promoted the migratory and invasive abilities of the A431-P and A431-III cells. Notably, the production of H₂O₂ was increased by the knockdown of MnSOD. One potential explanation for this is an increase in superoxide efflux from the mitochondria to the cytoplasm and the conversion of superoxide to H₂O₂ by CuSOD. Confirmation of this hypothesis requires further analysis.

Lu and Qu have been frequently reported as important potential anticancer and antioxidant agents. Lu has been demonstrated to activate catalase in HT-29 cells, CH27 cells and in a mouse model (27,45,46). Although the expression of MnSOD was increased by Lu in CH27 cells (27), the same effect was not observed in A431-P or A431-III cells.

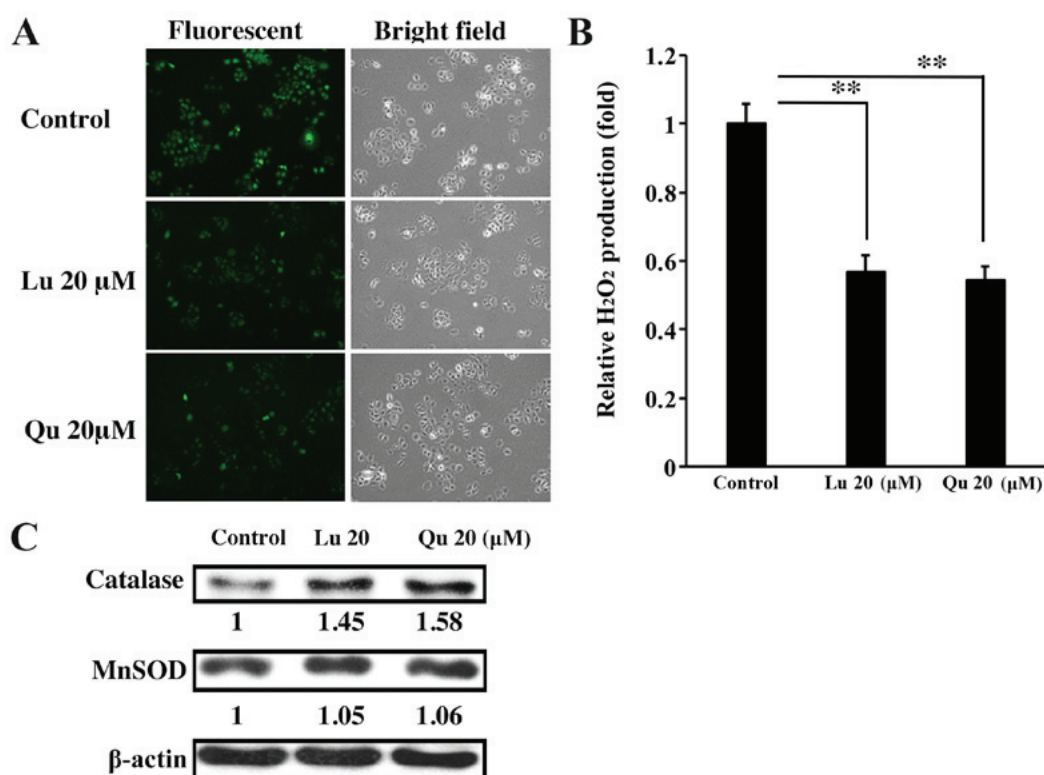


Figure 7. Lu and Qu reduce the production of H₂O₂ by increasing the expression of catalase. (A) A431-P and A431-III cells were treated with 20 μM of Lu and Qu for 24 h. The production of H₂O₂ in A431-P and A431-III cells was measured by using 2',7'-dichlorofluorescein diacetate and fluorescent microscope (magnification, x200), and (B) fluorescent intensity was detected using the Amplex[®] Red Hydrogen Peroxide/Peroxidase Assay kit. Statistical significance was determined by a one-way ANOVA with Tukey's test (**P*<0.01). (C) Protein levels of catalase and MnSOD were determined using western blot analysis. Lu, luteolin; Qu, quercetin; H₂O₂, hydrogen peroxide; MnSOD, manganese superoxide dismutase.

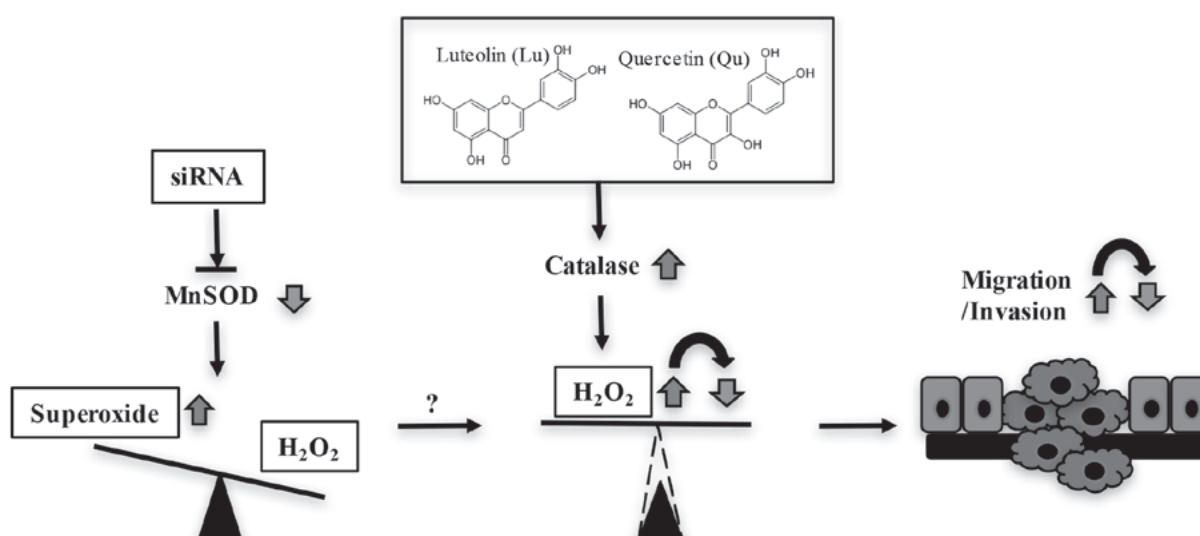


Figure 8. Schematic of the ROS imbalance induced by a reduction in MnSOD. A reduction in MnSOD increases the production of H₂O₂, which is induced by an unknown signaling mechanism. Luteolin and quercetin treatment increased the expression of catalase and reduce the production of H₂O₂. Subsequently, the migration and invasive ability of the invasive A431-III cells is reduced. ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; H₂O₂, hydrogen peroxide; siRNA, small interfering RNA.

Qu has been reported to restore the activity of catalase in a Huntington's disease mouse model (29), a diabetic rat model (47), in spermatozoa (48) and for neuroprotection in rat brains (49). These reports are consistent with the finding of the present study, whereby the expression of catalase was induced by Lu and Qu.

The data obtained in the present study indicate that the imbalance of ROS in A431-P and A431-III cells is due to reduced MnSOD activity, which subsequently promotes the metastatic ability of cancer cells. MnSOD may not be a suitable therapeutic target in squamous cell carcinoma; however, using antioxidant agents, such as Lu and Qu, to reduce H₂O₂

by increasing catalase activity, may be a useful therapeutic strategy (Fig. 8).

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Availability of data and materials

The data and materials used in the present study are available from the corresponding author on reasonable request.

Authors' contributions

WHH, MTL, CWL and CHC conceived and designed the study. JJF, HHH and WJZ performed the majority of the experiments and analyzed the data. TPK and YLAL assisted with manuscript preparation and data analysis. KCC assisted with RT-PCR analysis. CYC assisted with western blotting. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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