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Tumor-suppressive Maspin Functions as a Reactive Oxygen Species Scavenger

IMPORTANCE OF CYSTEINE RESIDUES[*S]

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Background: Maspin is a multifaceted serpin, but the role of intracellular maspin is still not well understood.
Results: Our study provides evidence that the structurally exposed cysteine residues in maspin reduce oxidative stress and cell proliferation.
Conclusion: Cysteine thiols in maspin scavenge reactive oxygen species in tumor cells.
Significance: These findings elucidate the importance of cysteine residues of maspin in curbing oxidative stress.

Maspin is a member of the serine protease inhibitor (serpin) superfamily and displays tumor-suppressing activity by controlling cell migration, proliferation, apoptosis, and adhesion. Here, we provide evidence that maspin acts as a reactive oxygen species (ROS) scavenger through oxidation of three structurally exposed cysteine thiols to sulfenic acid. Ablation of these cysteine residues in maspin resulted in a significant increase in total ROS production in mouse mammary TM40D cells. Also, cells containing a triple-cysteine mutant of maspin showed elevated ERK1/2 activity, a downstream target of ROS, and enhanced proliferation and colony formation. These findings establish a novel mechanism by which maspin utilizes its cysteine thiols to inhibit oxidative stress and cell growth.

Oxidative stress, a hallmark of many tumors, is caused by an imbalance between the generation of reactive oxygen species (ROS)[2] and cells' ability to clear oxidants. Processes associated with proliferation, apoptosis, and senescence may be the result of activation of signaling pathways in response to intracellular changes in ROS. Thus, excessive production and inadequacy in a normal cell's antioxidant defense system can cause the cell to experience oxidative stress (1, 2).

Maspin is a member of the serpin family that has tumor-suppressing activities (3, 4). It is well established that maspin is present in both extracellular and intracellular locations; however, the majority of maspin protein is located in the cytosol (5–7). In breast tumor cells, maspin overexpression sensitizes cells to undergo apoptosis (8). However, a high level of maspin expression in normal mammary epithelial cells does not easily induce apoptosis. This tumor-specific behavior of maspin prompted us to consider that intracellular maspin may have other cellular function(s), in addition to the apoptosis-inducing effects. Moreover, it has been reported that maspin interacts with enzymes of the GSH redox system such as GST (9) and glutathione peroxidase (10), suggesting that maspin might regulate cellular oxidative stress. Our laboratory has previously shown that in normal mammary epithelial MCF-10A cells, maspin forms an intramolecular disulfide bond when exposed to a high level of oxidative stress (hydrogen peroxide (H_2O_2)) (11), suggesting that maspin may regulate cellular ROS through its cysteine residues.

Among various protein modifications, much attention has recently been paid to the cysteine-targeted oxidation in regulation of protein function under stress conditions. Protein cysteine residues are highly susceptible to various types of oxidation. Sulfenic acid (–SOH) is the initial product of cysteine oxidation and is a key intermediate in the functional modulation of enzymes and proteins. Under variant biochemical circumstances, it may serve to mediate redox signaling (12, 13). Although sulfenic acid is likely to represent a dynamic and transient oxidation product, its unique chemistry allows it to be captured by dimedone-based labeling reagents before progressing to a potentially more complex array of disulfide-bonded or oxidized products (*e.g.* sulfinic acid ($–SO_2H$) and sulfonic acid ($–SO_3H$)) (14, 15)

Maspin has eight cysteine residues, which prompted us to explore cysteine-targeted oxidation of this multifaceted protein in the regulation of ROS metabolism. We found that only three cysteine residues, Cys-183, Cys-205, and Cys-323, are structurally fully exposed. Given the antioxidant capacity of the cysteine thiol group, we hypothesized that these exposed cysteine residues in maspin may act as potent scavengers/quenchers of ROS. In this study, we provide evidence that maspin indeed acts as a ROS scavenger. Maspin-overexpressing cells are more resistant to oxidative stress, and this property is attributed to the cysteine residues in maspin.

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[S] This article contains supplemental Tables 1 and 2.
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[2] The abbreviations used are: ROS, reactive oxygen species; KO, knock-out; HE, hydroethidine; STS, staurosporine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; pERK1/2, phosphorylated ERK1/2.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Cell Culture—Maspin x-ray crystal structures were reported by Law *et al.* (16) and Al-Ayyoubi *et al.* (17). We used Molsoft ICM-Pro version 3.48 to analyze the x-ray structure of human maspin (Protein Data Bank code 1XU8) reported by Law *et al.* (16) to analyze the presence of exposed cysteine residues, which can serve as site for oxidation. The QuikChange II site-directed mutagenesis kit (Stratagene) was used to mutate cysteine residues to serine residues in pEF-IRES-neo-h.maspin using specific mutagenic primers (supplemental Table 1). Mutations in the plasmid were confirmed by DNA sequencing.

Murine mammary tumor TM40D cells were used and maintained as described previously (18). TM40D cells were transfected with pEF-IRES-neo-h.maspin (wild-type ($TM40D^{Mp}$) or triple-mutant ($TM40D^{T}$)) or with the control vector alone ($TM40D^{Neo}$) by Effectene reagent (Qiagen). The stable transfectants were selected with G418 medium (600 μg/ml) for 14 days, and expression of maspin was confirmed by Western blot analysis. The GST fusion proteins (GST-tagged wild-type ($GST\text{-}Mp^{WT}$) and triple-mutant ($GST\text{-}Mp^{T}$) human maspin) were induced by isopropyl β-D-thiogalactopyranoside (1 mM) and purified using glutathione-agarose (Sigma). Thrombin was used to cleave the maspin from the agarose beads. The size and purity of the proteins were confirmed by SDS-PAGE and Western blot analysis.

Human mammary tumor and immortalized epithelial cells (MCF-7, MCF-10A, and MCF-10A229) were maintained as described previously (19). In a previous study, we showed that homozygous maspin knock-out (KO) mice are embryonically lethal (20); therefore, we isolated mouse primary mammary epithelial cells from WT and heterozygous maspin KO mice as described previously (21).

Western Blot Analysis—Cell lysates were prepared in radioimmune precipitation assay buffer with protease inhibitor mixture (Thermo Scientific). Cellular debris was cleared from lysates by centrifugation, and protein concentration was determined by the BCA protein assay (Pierce). Samples were separated by 10% SDS-PAGE, transferred to a PVDF membrane (GE Healthcare), and blotted with rabbit anti-maspin antibody AbS4A (3) and anti-actin antibody (Sigma A2066). HRP-labeled goat anti-rabbit polyclonal antibody was used as a secondary antibody, and proteins were visualized with enhanced chemiluminescence substrate (Pierce).

Quantification of ROS—The fluorogenic substrate carboxy-2′,7′-dichlorodihydrofluorescein diacetate was used to detect intracellular ROS (22). Briefly, 10^6 cells in a 6-well dish were plated and incubated overnight at 37 °C. The next day, the culture medium was discarded, and cells were washed twice with PBS, followed by incubation with 20 μM carboxy-2′,7′-dichlorodihydrofluorescein diacetate at 37 °C for 30 min in serum-free medium. Cells were either left untreated or treated with different ROS inducers at the indicated final concentrations and incubated at 37 °C for the indicated time periods. At the end of the exposure period, cell supernatants were discarded, and cells were washed with PBS and harvested using trypsin/EDTA. Cells were transferred to FACS tubes, and 20,000 events were analyzed using a Beckman Coulter Epics XL analyzer with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Results are depicted as -fold change in fluorescence intensity.

Measurement of Superoxide ($O_2^{\cdot-}$) Levels—To measure superoxide levels in the cell culture, we used 2,7-diamino-10-ethyl-9-phenyl-9,10-dihydrophenanthridine, 3,8-diamino-5,6-dihydro-5-ethyl-6-phenylphenanthridine, and hydroethidine (HE), a fluorogenic probe that is widely used to detect superoxide levels (23). $TM40D^{Neo}$ and $TM40D^{Mp}$ cells were treated with staurosporine (STS; 1 μM) for 3 h and incubated with 10 μM HE for 1 h. Treating cultured cells with STS is known to induce a rapid and prolonged increase in ROS (24, 25). Cells were washed twice with cold PBS, scraped, and kept for 30 min on ice. The cell suspension was centrifuged at 1000 × *g* for 5 min at 4 °C. Pellets were either stored at −80 °C or immediately processed for HPLC analysis. Cells were lysed using 0.1% Triton X-100 in Dulbecco's PBS. Cell lysates were mixed with an equal amount of 0.2 M solution of $HClO_4$ in MeOH and left undisturbed for 2 h to allow protein precipitation. Samples were centrifuged at 20,000 × *g* for 30 min at 4 °C, and 100 μl of supernatant was mixed with an equal amount of a 1 M solution of potassium phosphate buffer (pH 2.6). After centrifugation at 20,000 × *g* for 15 min at 4 °C, samples were analyzed by HPLC-MS. The instrumental setup for the analysis of HE and its oxidation products by HPLC-MS is depicted in supplemental Table 2. HPLC peak areas were normalized to protein concentration.

Cell-Extracellular Matrix Adhesion Assay—Adhesion of mouse mammary $TM40D^{Neo}$ cells to Matrigel matrix (BD Biosciences) was used to determine the biological activity of recombinant proteins. The adhesion assay was performed as described by Corbett *et al.* (26). Briefly, cells were suspended in medium containing 100 nM maspin ($GST\text{-}Mp^{WT}$ and $GST\text{-}Mp^{T}$) or GST and then cultured on a Matrigel-coated 96-well plate at a density of 5×10^4 cells/well in triplicates for 4 h. The plates were washed twice with PBS and incubated with 50 μl of hexosaminidase substrate (3.75 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide, 0.25% Triton X-100, and 0.05 M citrate buffer (pH 5)) for 1.5 h at 37 °C. After incubation, 75 μl of development buffer (5 mM EDTA and 50 mM glycine (pH 10.4)) was added, and readings were recorded at 405 nm.

Measurement of GSH/GSSG Ratios—A luminescence-based system was used for detection and quantification of GSH/GSSG ratios in cultured cells (Promega V6611).

Detection of Protein Sulfenic Acid Modifications—Detection of protein sulfenic acid *in vitro* was done as described previously (14, 15, 27). Briefly, equal amounts (62.5 ng) of thrombin-cleaved recombinant Mp^{WT} and Mp^{T} were oxidized with H_2O_2 (1 mM) for 1 min in the presence of dimedone (10 mM). The oxidation of cysteine residues was detected by Western blot analysis using an antibody (Millipore 07-2139) that specifically recognizes dimedone-derivatized cysteine sulfenic acid residues.

Trapping of Sulfenic Acid in Cells (Immunoprecipitation)—Cells were first treated with H_2O_2 (250 μM) for 3 h and then lysed in lysis buffer (100 mM Tris (pH 7.4), 1% Triton X-100, and protease inhibitor mixture) with or without dimedone (10 mM)

for 45 min before the addition of *N*-ethylmaleimide to a final concentration of 100 mM for another 10 min. Cellular debris was cleared from lysates by centrifugation, and protein concentration was determined by the BCA protein assay. Whole cell extracts (1 mg) were incubated overnight (constant rocking) with 0.5 μg of rabbit anti-maspin antibody AbS4A (3) or control rabbit IgG at 4 °C. Protein A-Sepharose-coupled beads (Amersham Biosciences) were added and incubated for 1 h at 4 °C under constant agitation. Beads were centrifuged, washed briefly with ice-cold lysis buffer, and finally incubated with elution buffer for 15 min at room temperature. Samples were mixed with 5× nonreducing buffer; separated on SDS-polyacrylamide gels; transferred to a PVDF membrane; and probed for maspin, cysteine sulfenic acid, and IgG. Appropriate secondary antibodies were added, and proteins were visualized with enhanced chemiluminescence substrate. The anti-cysteine sulfenic acid antibody (Millipore 07-2139) used recognizes proteins containing dimedone-bound cysteine sulfenic acid.

Soft Agar Colony Formation Assay—As described previously (28), 6-well dishes were plated with the bottom agar (0.7% agarose) for 30 min. Cells were mixed with the top agar (0.3% agarose) at a concentration of 5×10^4 cells/well and allowed to solidify. The cells were fed every fifth day with medium and grown at 37 °C for 3 weeks. Cells were stained for 1 h with 0.05% crystal violet, and the numbers of purple-colored colonies were counted as 10 fields/well at ×40 using ImageJ software with a cutoff range of 20–5000 pixels.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) in Vitro Cell Proliferation Assay—Cells were seeded at 10^3 cells/well in a 96-well plate and allowed to grow at 37 °C with 5% CO_2. At each time point, MTT reagent (5 mg/ml) was added in a volume of 10 μl/well and incubated at 37 °C with 5% CO_2 for 3 h. The medium was aspirated, and 100 μl of Me_2SO was added and mixed until a uniform purple color was formed. The cell samples were measured using a plate reader at 570 nm. Assays were performed in triplicates.

ERK1/2 Phosphorylation—Protein lysates (30 μg) were separated by 12% SDS-PAGE, and a PhosphoPlus kit (Cell Signaling Technology 9100) was used to determine ERK1/2 phosphorylation.

Statistical Analysis—All experiments were carried out three times or as stated. Quantification of Western blots was performed using Scion Image. Statistical analysis (two-tailed *t* test) was based on a minimum of three replicates using Prism statistical software. The differences were considered significant if $p < 0.05$.

RESULTS

Status of Intracellular ROS Levels in Mouse Primary Mammary Epithelial Cells Isolated from WT and Heterozygous Maspin KO Mice and Maspin-silenced Human Immortalized Mammary Epithelial MCF-10A Cells—To understand the role of maspin in oxidative stress, we isolated primary mammary epithelial cells from WT and heterozygous maspin KO mice and measured their ROS levels. Fig. 1*A* depicts the Western blot analysis for maspin expression in cells isolated from WT and heterozygous maspin KO mice. We observed that the cells isolated from maspin KO mice had significantly higher intracellular ROS levels compared with WT mice ($p < 0.001$) (Fig. 1*B*). We also observed a significant increase in ROS production in KO cells compared with WT cells when they were pretreated with H_2O_2 for 3 h ($p < 0.05$) (Fig. 1*C*). To further analyze the importance of maspin in ROS production, we used maspin-silenced MCF-10A cells (MCF-10A229), which, as confirmed by Western blot analysis, showed attenuated levels of maspin (Fig. 1*D*). MCF-10A cells displayed significantly lower levels of ROS compared with maspin-silenced MCF-10A229 cells ($p < 0.01$) (Fig. 1*E*). Collectively, these results indicate that the presence of maspin in primary and immortalized mammary epithe-

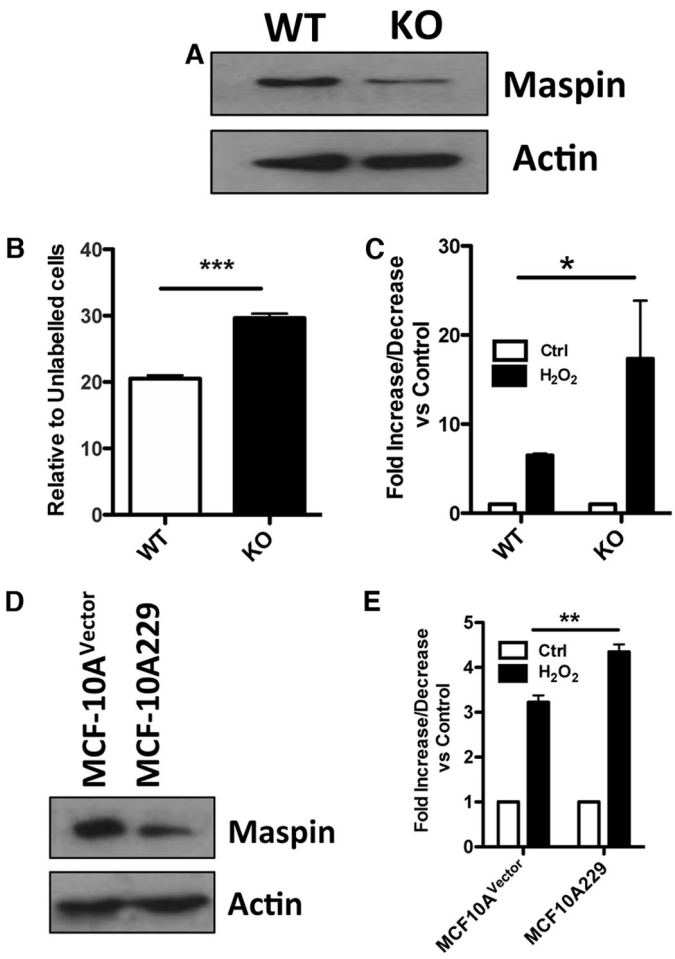

FIGURE 1. **Total intracellular ROS levels in primary mammary epithelial cells isolated from WT and heterozygous maspin KO mice and maspin-silenced human immortalized mammary epithelial MCF-10A cells.** Total intracellular ROS levels were measured by flow cytometric analysis using carboxy-2′,7′-dichlorodihydrofluorescein diacetate as a fluorogenic substrate. *A*, Western blot analysis of maspin expression in primary mammary epithelial cells isolated from WT and heterozygous maspin KO mice. Equal amounts of proteins (25 μg/lane) were loaded and immunoblotted for maspin and actin (a loading control). *B*, constitutive levels of ROS in primary mammary epithelial cells isolated from WT and heterozygous maspin KO mice ($n = 3$). *C*, ROS levels in primary mammary epithelial cells isolated from WT and heterozygous maspin KO mice ($n = 3$) after treatment with H_2O_2 (250 μM) for 3 h. *Ctrl*, control. *D*, Western blot analysis of maspin expression in human immortalized mammary epithelial MCF-10A and MCF-10A229 cells. *E*, ROS levels in human immortalized mammary epithelial MCF-10A and MCF-10A229 (maspin-silenced) cells after treatment with H_2O_2 (250 μM) for 3 h. Data shown are means ± S.D. of three independent experiments. *Asterisks* indicate significance according to a two-tailed *t* test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

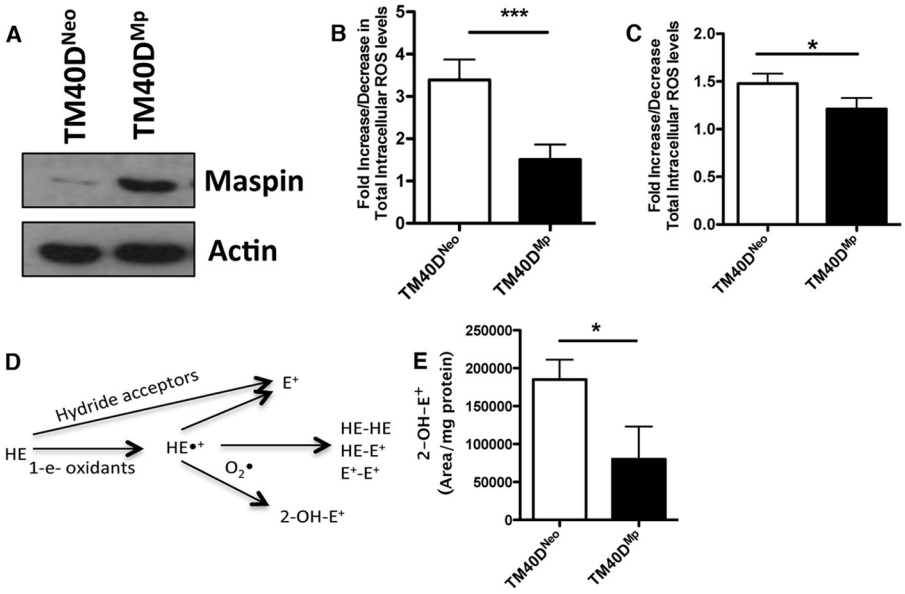

FIGURE 2. **Maspin-overexpressing tumor cells are resistant to oxidative stress.** *A*, Western blot analysis of maspin expression in TM40D^{Neo} and TM40D^{Mp} cells. Equal amounts of proteins (25 μg/lane) were loaded and immunoblotted for maspin and actin (a loading control). *B*, ROS levels in TM40D^{Neo} and TM40D^{Mp} cells after treatment with H_2O_2 (250 μM) for 3 h. *C*, ROS levels in TM40D^{Neo} and TM40D^{Mp} cells after treatment with antimycin A (100 μM) for 1 h. *D*, schematic representation of oxidation of HE and formation of various oxidation products. 2-Hydroxyethidium (*2-OH-E*$^+$) is the primary product of the reaction of HE and superoxide (O_2^-), and other products such as E$^+$ and dimers of HE (*HE-HE*, *HE-E*$^+$, and *E*$^+$-*E*$^+$) are indicators of one-electron oxidants. *E*, levels of 2-hydroxyethidium (2-OH-E$^+$) as an indicator of superoxide (O_2.) in TM40D^{Neo} and TM40D^{Mp} cells after treatment with STS (1 μM) for 3 h as measured by HPLC-MS. The results are shown as the area under the peak/mg of protein. Data shown are means ± S.D. of three independent experiments. *Asterisks* indicate significance according to a two-tailed *t* test: *, $p < 0.05$; ***, $p < 0.001$.

lial cells makes them more efficient to quench ROS and therefore indicate the importance of maspin in ROS regulation.

Maspin-overexpressing Cells Are Resistant to Oxidative Stress—To directly target the importance of maspin in oxidative stress, we used the mouse mammary epithelial cell line TM40D, which expresses low levels of maspin, as a cellular model (Fig. 2*A*). Maspin was overexpressed in TM40D cells (Fig. 2*A*), and total intracellular ROS levels were measured. Our results demonstrate that exposure for 3 h to 250 μM H_2O_2 was sufficient to induce a significant increase in intracellular ROS in TM40D^{Neo} cells compared with maspin-overexpressing TM40D^{Mp} cells ($p < 0.001$) (Fig. 2*B*).

To determine the physiological relevance or the effect of endogenous ROS on mammary epithelial cells, we used antimycin A, a compound that inhibits electron transport at complex III of the mitochondrial respiratory chain, thereby inducing the production of superoxide and other ROS in the cells (29, 30). Treating cells with antimycin A resulted in decreased ROS levels in maspin-overexpressing cells ($p < 0.05$) (Fig. 2*C*).

In addition, HE was used to determine the O_2^- levels as an indicator of oxidative stress in response to an alternative ROS inducer, *i.e.* STS, by HPLC-MS in TM40D^{Neo} and TM40D^{Mp} cells. Fig. 2*D* depicts the schematic representation of the reaction of HE and superoxide radical anion (O_2^-) to form 2-hydroxyethidium. We observed a significant decrease in the levels of superoxide in maspin-overexpressing cells compared with TM40D^{Neo} cells in response STS treatment ($p < 0.05$) (Fig. 2*E*).

Cysteine Residues in Maspin Control Oxidative Stress—Having established that maspin is involved in modulating oxidative stress in normal mammary epithelial and cancer cells, we further sought to identify the underlying mechanism of maspin action. Due to the presence of an active thiol (–SH) group, cysteine residues play an important role as oxidative stress sensors in various proteins (12, 13). Using Molsoft ICM-Pro version 3.48, we analyzed the x-ray structure of human maspin protein (Protein Data Bank code 1XU8) reported by Law *et al.* (16). We identified that of eight cysteine residues present in human maspin, only three residues at positions 183, 205, and 323 are structurally exposed and might serve as possible sites for oxidation. In contrast, cysteine residues at positions 20, 34, 214, 287, and 373 are buried. Previously, it has been shown that mutating all of the cysteines to serine/alanine by site-directed mutagenesis does not change the structure of maspin (17). Therefore, we mutated either single cysteine residues (Cys-183 or Cys-323) or all three exposed cysteine residues to serine. Fig. 3*A* shows the three-dimensional structure of maspin with the three structurally exposed cysteine residues (Cys-183, Cys-205, and Cys-323). TM40D cells were transfected with different maspin cysteine mutants, and expression of maspin was confirmed by Western blot analysis (Fig. 3*B*).

Mutating a single cysteine residue, *i.e.* Cys-183 or Cys-323, individually in maspin led to a 2-fold increase in ROS levels in comparison with TM40D^{Mp} cells (Fig. 3*C*), suggesting that both of these cysteine residues are equally important in regulating cellular ROS. When all three surface cysteines were mutated, the cellular ROS level in TM40D^{T} cells was further increased (~1.4-fold) compared with TM40D^{C183S} or TM40D^{C323S} cells ($p < 0.05$) (Fig. 3*C*). Increased ROS levels in TM40D^{T} cells correlated with the absence of surface-exposed cysteine residues, thereby establishing the importance of maspin cysteine residues in modulating ROS.

Enzymes such as those involved in the glutathione redox cycle play an important role in detoxification of various oxidant species (31). A decreased GSH/GSSG ratio is considered indic-

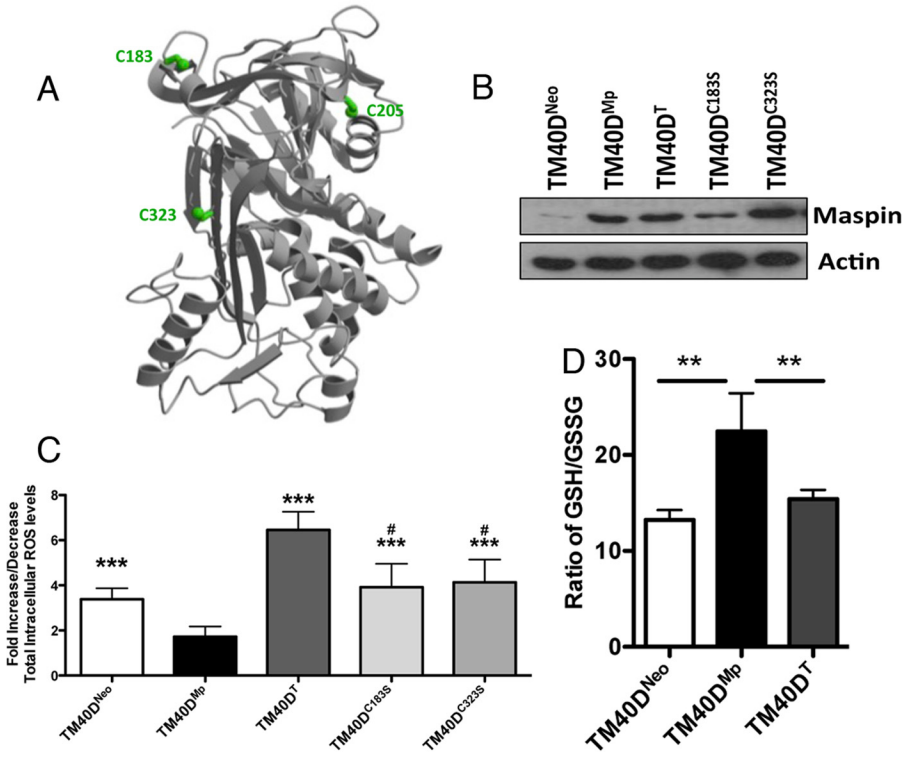

FIGURE 3. **Importance of cysteine residues and role in oxidative stress.** *A*, three-dimensional structure of maspin highlighting the three cysteine residues (*green*) that were mutated to serine at positions 183, 205, and 323. *B*, Western blot analysis of maspin expression in TM40D^{Neo}, TM40D^{Mp}, TM40D^{T}, and other mutant maspin cells. Equal amounts of proteins (25 μg/lane) were loaded and immunoblotted for maspin and actin (a loading control). *C*, comparison of total intracellular ROS levels in various TM40D cells treated with H_2O_2 (250 μM) for 3 h as determined by flow cytometric analysis. *D*, GSH/GSSG ratio in TM40D^{Neo}, TM40D^{Mp}, and TM40D^{T} cells. Data shown are means $\pm$ S.D. of three independent experiments. *Asterisks* indicate significance according to a two-tailed *t* test: **, $p < 0.01$ compared with TM40D^{Mp}; ***, $p < 0.001$ compared with TM40D^{Mp}; #, $p < 0.05$ compared with TM40D^{T}.

ative of increased oxidative stress (32, 33). We found that overexpression of maspin in TM40D cells resulted in an increased GSH/GSSG ratio ($p < 0.01$) (Fig. 3*D*). However, expressing triple-mutant maspin in TM40D cells resulted in a decreased GSH/GSSG ratio compared with cells expressing WT maspin in the absence of ROS stimulus ($p < 0.01$) (Fig. 3*D*).

Triple-mutant Maspin Is Biologically Active and Evidence for Oxidation of Cysteine Thiol to Sulfenic Acid—To confirm that mutant maspin retains its normal biological activity, we tested whether the triple-mutant recombinant maspin (GST-Mp^{T}) could increase cell adhesion to the extracellular matrix similar to the WT maspin protein (GST-Mp^{WT}). Increased adhesion of TM40D^{Neo} cells to Matrigel matrix was observed when cells were pretreated with GST-Mp^{T} compared with its control ($p <$ 0.01) (Fig. 4*A*).

To elucidate whether selected cysteine residues of maspin trap free radicals or other oxidants, WT and triple-mutant recombinant maspin proteins were treated with H_2O_2 in presence or absence of dimedone. Dimedone is a highly specific agent that reacts with cysteine sulfenic acid (14, 15). Sulfenic acid is the initial oxidation product, which subsequently reacts with dimedone to form a stable covalent bond (Fig. 4*B*) (15). Probing with anti-cysteine sulfenic acid antibody showed an absence of signal in lanes loaded with triple-mutant maspin compared with WT maspin (Fig. 4*C*). These results clearly demonstrate oxidation of thiol groups to cysteine sulfenic acid in maspin.

Moreover, to prove the participation of cysteine residues in maintaining the redox state in cells, we immunoprecipitated maspin from H_2O_2-treated TM40D^{Mp}, TM40D^{C183S}, TM40D^{C323S}, and TM40D^{T} cells in the presence or absence of dimedone, and the immunoprecipitated products were analyzed for the presence of dimedone-derivatized cysteine sulfenic acid residues under nondenaturing conditions. Fig. 4*D* shows the presence of maspin cysteine sulfenic acid in TM40D^{Mp}, TM40D^{C183S}, and TM40D^{C323S} cells but not in TM40D^{T} cells. These results further demonstrate the importance of structurally exposed cysteine residues. Similar results were obtained when the experiment was performed with human mammary cancer epithelial MCF-7 cells (Fig. 4*D*, *lower panel*), which constitutively express maspin.

Cysteine Residues Affect Cell Proliferation Pattern of TM40D Cells via Activation of ERK1/2—Previously, we and others have shown that maspin suppresses tumor growth, invasion, and metastasis of breast and prostate cancer (35, 36). To determine the effect of overexpression of maspin and cysteine mutations on the tumorigenic properties of TM40D cells, we assessed the anchorage-independent growth and *in vitro* proliferation of WT and mutant maspin TM40D cells using soft agar colony formation and MTT assays, respectively. Anchorage-independent growth is one of the hallmarks of transformation, and the soft agar assay is a commonly used *in vitro* assay for detecting malignant transformation of cells (28, 37). The abilities of the cells to form colonies on soft agar are shown in Fig. 5*A*. Com-

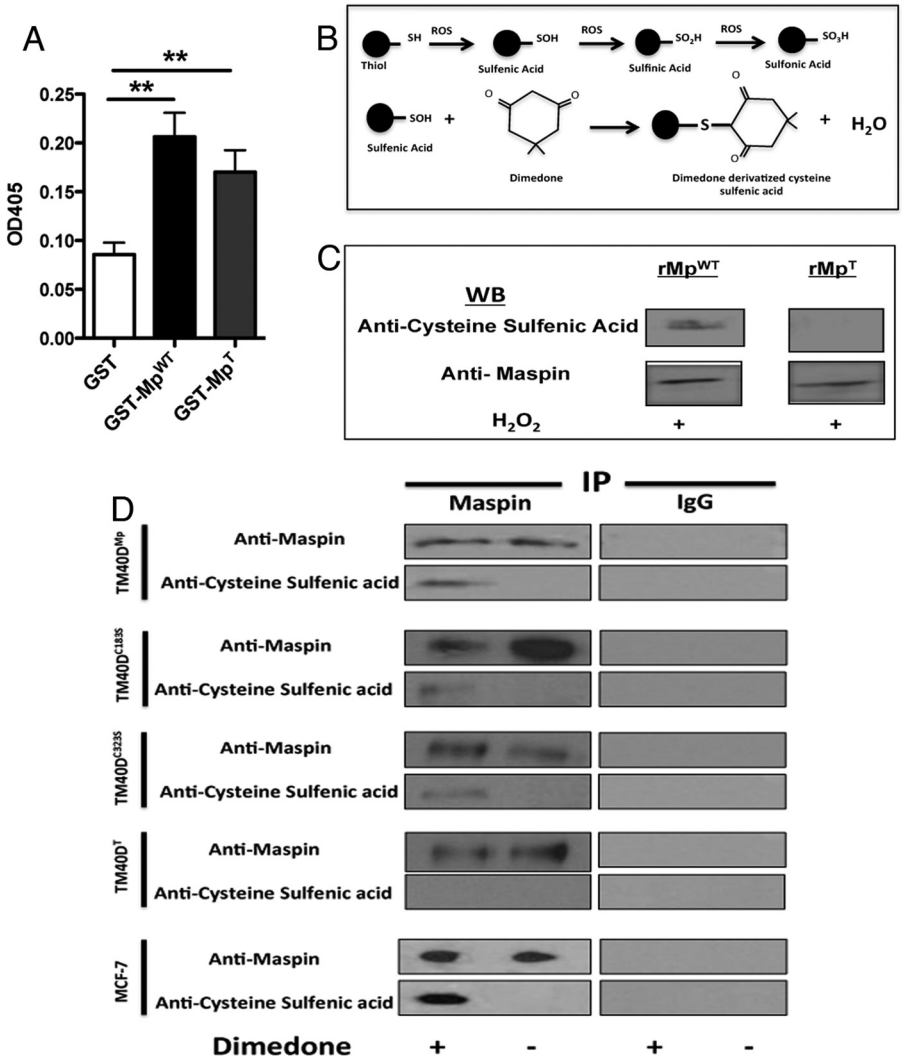

FIGURE 4. **Triple-mutant maspin is biologically active and evidence for oxidation of cysteine to sulfenic acid.** *A*, mutant maspin retains the ability to enhance cell adhesion to the extracellular matrix. Increased adhesion of TM40D^{Neo} cells to Matrigel matrix was observed when cells were pretreated with WT and triple-mutant recombinant maspin (100 nM) compared with its GST control. *B*, biochemistry of oxidation of thiol groups (–SH) and capturing of sulfenic acid (–SOH) using dimedone. Protein thiols (–SH), which are susceptible to oxidation by ROS, generate sulfenic acid (–SOH) and largely irreversible $–SO_2H$ and $–SO_3H$. Sulfenic acid can be labeled by dimedone or dimedone-based chemicals. *C*, equal amounts (62.5 ng) of WT and triple-mutant recombinant (*r*) maspin were oxidized with H_2O_2 (10 mM) for 1 min in the presence of dimedone (10 mM), and oxidation of cysteine residues was detected by Western blot (*WB*) analysis using an antibody that specifically recognizes dimedone-derivatized cysteine sulfenic acid residues. *D*, cells were treated with H_2O_2 (250 μM) for 3 h and then lysed in the presence or absence of dimedone (10 mM). Maspin was immunoprecipitated (*IP*) and immunoblotted with anti-maspin antibody and an antibody that specifically recognizes dimedone-derivatized cysteine sulfenic acid residues. Data shown are means ± S.D. of three independent experiments. **, $p < 0.01$ (two-tailed *t* test).

pared with the vector-transfected cells, maspin-overexpressing TM40D^{Mp} cells formed significantly fewer colonies/well ($p < 0.01$) (Fig. 5*A*), a feature indicative of a decrease in anchorage-independent growth, whereas no significant difference was observed between the vector control and the triple-mutant maspin ($p > 0.05$). A significant increase in colony formation was observed between TM40D^{Mp} and TM40D^{T} cells ($p < 0.01$). The results of the MTT proliferation assay revealed that WT maspin-overexpressing TM40D^{Mp} cells had a low proliferation rate compared with TM40D^{Neo} and TM40D^{T} cells over a time period of 96 h (Fig. 5*B*). To obtain direct evidence that the deviation in the proliferation pattern of TM40D^{Neo} and TM40D^{T} cells was due to changed ROS levels, we treated both types of cells with *N*-acetylcysteine (NAC), a strong antioxidant. We observed a significant drop in the proliferation rate of TM40D^{Neo} and TM40D^{T} cells upon NAC treatment, whereas no significant change was observed in TM40D^{Mp} cells (Fig. 5*C*). Thus, these results provide strong evidence linking a high proliferation rate to elevated intracellular ROS levels.

Increased oxidative stress has been shown to cause proliferation defects in cells via targeting key signaling molecules such as ERK1/2 (38). The total ERK1/2 MAPK level was found to be nearly constant in all three TM40D-derived cell lines by Western blot analysis (Fig. 5*D*). However, the corresponding activated phosphorylated forms (pERK1/2) were found to be consistently increased in TM40D^{Neo} and TM40D^{T} cells compared with TM40D^{Mp} cells (Fig. 5*D*), suggesting that TM40D^{Neo} and TM40D^{T} cells have higher constitutive expression of activated pERK1/2 than TM40D^{Mp} cells. Treating cells with the antioxidant NAC significantly reduced the levels of activated pERK1/2

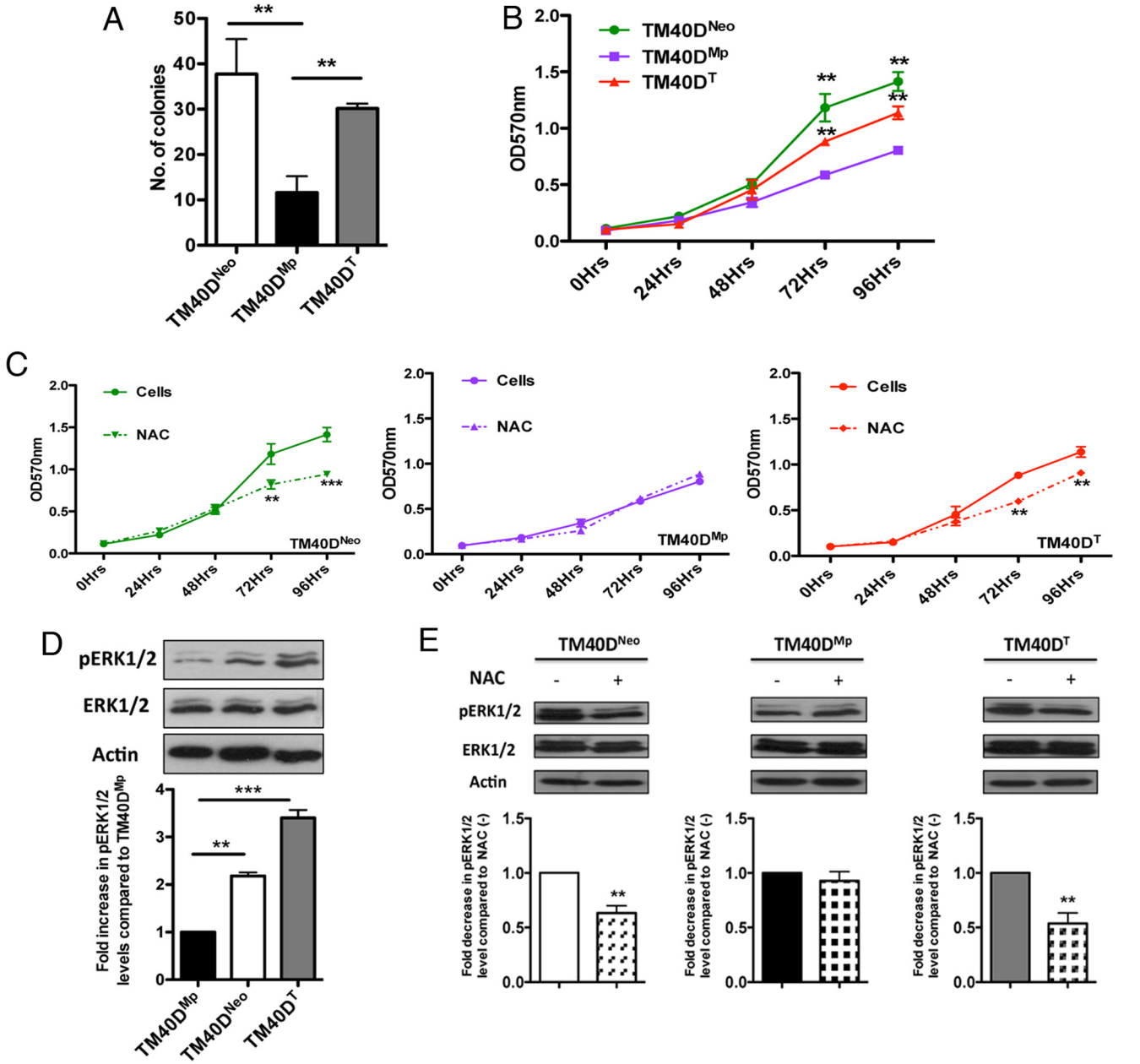

FIGURE 5. **Cysteine mutant cells have increased cell proliferation and ERK1/2 activation.** *A*, number of colonies formed *in vitro* in different cell lines using the soft agar colony formation assay. *B*, comparison of cell proliferation patterns in three different cell lines as determined by the MTT assay. *C*, treatment with antioxidant (*i.e.* NAC) significantly decreases proliferation of $TM40D^{Neo}$ and $TM40D^{T}$ cells. *D*, comparison of pERK1/2 levels in $TM40D^{Neo}$ and $TM40D^{T}$ cells compared with $TM40D^{Mp}$ cells. *E*, effect of NAC treatment on pERK1/2 levels in $TM40D^{Neo}$, $TM40D^{Mp}$, and $TM40D^{T}$ cells. Representative Western blots are shown above the bar graphs, and each lane corresponds to the respective bars. Data are shown as means ± S.D. of three independent experiments. *Asterisks* indicate significance according to a two-tailed *t* test: **, $p < 0.01$; ***, $p < 0.001$.

(Fig. 5*E*) and the cell proliferation rate in $TM40D^{Neo}$ and $TM40D^{T}$ cells; however, no change was observed in $TM40D^{Mp}$ cells. These results clearly demonstrate that maspin inhibits ERK1/2 activities, likely through ROS scavenging, and that this mechanism controls epithelial cell proliferation.

DISCUSSION

Oxidative damage to any cellular constituent can lead to disease development if remained unchecked (1, 39). Maspin belongs to the serpin family of non-inhibitory protease inhibitors (3) and is abundantly produced in normal mammary luminal epithelial and myoepithelial cells (3, 40). Our laboratory (4, 18) and others (8, 41) have demonstrated that maspin acts as a multifaceted protein, interacting with extra- and intracellular groups of proteins and regulating key functions of cell adhesion, motility, apoptosis, and angiogenesis. It is also critical in mammary gland development (4, 18, 41). The role of maspin in oxidative stress has been speculated in the last few years (9, 11). In this study, we have provided evidence, for the first time, of the oxidation of cysteine residues in maspin. Additionally, we have demonstrated that maspin acts as a ROS scavenger to provide resistance against oxidative stress. This suggests a new paradigm that maspin controls tumor cells to proliferate in an environment of oxidative stress by maintaining redox homeostasis.

Our data also show that surface-exposed cysteines regulate ROS as an intracellular serpin. The results of the adhesion assay (Fig. 4*A*) clearly demonstrate that this function of ROS regulation is independent of maspin's extracellular function, which increases cell adhesion to the extracellular matrix when maspin is secreted.

Our results (Fig. 1) indicate that cells expressing maspin have low levels of ROS due to the active participation of maspin in scavenging oxidants. The coordinated action of various cellular antioxidants in mammalian cells is critical for maintaining a steady redox state. Also, evidence suggests that maspin expression is regulated in response to redox status. Overexpression of manganese-containing superoxide dismutase leads to increased maspin expression (42, 43). This reinforces involvement of maspin in the regulation of cellular redox homeostasis.

It can be speculated that under lower oxidative stress or normal physiological conditions, different oxidative agents may also modify maspin cysteines. We therefore tested whether the maspin expression level in normal mammary epithelial cells or in cells with a reduced level of maspin (*e.g.* MCF-10A229) regulates cellular redox status. We then reintroduced maspin into tumor cells that do not express maspin to study the mechanism of maspin-mediated regulation of cellular ROS. A reduction in total intracellular ROS (Fig. 2*B*) in maspin-overexpressing mouse mammary epithelial cells (TM40D) further substantiates the importance of maspin in maintaining the redox state of cells. Increasing ROS in the mitochondria (antimycin A treatment) shows the physiological relevance (Fig. 2*C*) of maspin in oxidative stress, which is consistent with our finding that maspin attenuates ROS species such as superoxide ($O_2^{\cdot-}$) in STS-stressed TM40D cells (Fig. 2*E*). Taken together, our results indicate that the presence of maspin in mammary cells makes them more efficient in fighting against ROS irrespective of their origins: whether they are derived from mouse tumor epithelial cells (TM40D), mouse primary epithelial cells, or human mammary epithelial MCF-10A cells.

Emerging evidence suggests that redox-sensitive cysteine residues in proteins may function as oxidant sensors (44, 45). The conversion of these residues to sulfenic acid has been demonstrated in redox signaling in yeast, in T-cell activation, and in other proteins (14, 15, 45), providing strong support for the increasing roles of this modification in biology. Mutating three cysteine residues in maspin resulted in a significant increase in intracellular ROS levels in $TM40D^{T}$ cells (Fig. 3*C*). Furthermore, the levels of other antioxidant proteins were also found to be altered in cells expressing cysteine-to-serine mutant maspin ($TM40D^{T}$) compared with cells expressing WT maspin ($TM40D^{Mp}$). Our data suggest that these cysteines have a cumulative effect because mutation of either C183S or C323S led to similar significant increases in cellular ROS levels. Cells with triple-mutant maspin had an ~1.4-fold increase in ROS levels compared with cells with a single-cysteine mutation (Fig. 3*C*). However, there is still a possibility that certain redox agents may preferentially attack a particular cysteine in maspin *in vivo* depending on their size and structure. Future studies to determine whether certain redox agents affect different cysteines in maspin with different abilities to regulate cellular ROS are needed.

Besides its importance in redox homeostasis, overexpression of maspin also inhibits cancer cell proliferation. Maspin-overexpressing cells have reduced capacities to form colonies and to grow in soft agar. Mutation of cysteine residues resulted in a phenotype that formed more colonies (Fig. 5*A*). The results of the MTT assay revealed that $TM40D^{Neo}$ cells proliferated more rapidly than WT maspin-overexpressing cells. Increased proliferation of $TM40D^{Neo}$ cells coincided with increased ROS levels in these cells (Figs. 2*B* and 5*B*). Cells with mutant maspin ($TM40D^{T}$) also proliferated more rapidly compared with $TM40D^{Mp}$ cells (Fig. 5*B*). In agreement with this, Cia *et al.* (46) have also reported decreased proliferation rates with maspin overexpression in esophageal carcinoma. Also, NAC treatment reversed the oxidative stress phenotype of maspin-deficient or mutant maspin cells, thus indicating that proliferation is a result of increased ROS in these cells. ROS can activate kinases and/or inhibit phosphatases, resulting in stimulation of signaling pathways such as ERK1/2 (MAPKs) that are important in cell proliferation, differentiation, invasion, and apoptosis (47). In this study, we found higher levels of pERK1/2 in $TM40D^{Neo}$ and $TM40D^{T}$ cells compared with $TM40D^{Mp}$ cells (Fig. 5*D*). Antioxidant pretreatment led to a decrease in pERK1/2 expression in these cells (Fig. 5*E*). These observations suggest that increased oxidative stress activates ERK1/2, which in turn increases proliferation. These results collectively substantiate that maspin-overexpressing cells have lower ROS levels and therefore maintain lower pERK1/2 levels and proliferation rates compared with cell lines that have attenuated or mutant maspin expression.

This study indicates that under oxidative stress, cysteine thiols (–SH) in maspin are oxidized to cysteine sulfenic acid (–SOH), which may further interact with GST (9) or some other unidentified molecule(s) that reduces the oxidized thiol in the cells (*e.g.* glutathione, peroxiredoxin, etc.). Reducing –SOH to –SH in maspin makes it available for recycling and for scavenging more ROS in the intracellular microenvironment. Increased oxidative stress in cells with triple-mutant maspin demonstrates the absence of the above-proposed mechanism. Cells expressing WT maspin have a low proliferation rate, which is attributed to low ROS levels, and therefore suppressed tumor growth. In conclusion, maspin overexpression leads to resistance against oxidative stress, and maspin cysteine residues play an important role in maintaining the redox status of cells. Future studies will be aimed at determining the precise mechanism by which maspin regulates cellular ROS levels.

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