Tumor suppressive maspin functions as a ROS scavenger: Importance of cysteine residues

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Running Title: Maspin as a ROS scavenger

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Key Words: maspin; cysteine thiols; sulfenic acid; oxidative stress

Capsule
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 cellular 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.

SUMMARY
Maspin, a member of the serine protease inhibitor (serpin) superfamily, 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 results in a significant increase in total ROS production in TM40D mouse mammary cells. Also, the cells containing triple cysteine mutant of maspin show 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.

INTRODUCTION
Oxidative stress, a hallmark of many tumors, is caused by an imbalance between the generation of reactive oxygen species (ROS) 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 anti-oxidant 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, high level of maspin expression in normal mammary epithelial cells does not easily induce apoptosis. This tumor specific behavior of maspin prompted us to think 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 glutathione (GSH) redox system like glutathione S-transferase (GST) (9) and glutathione peroxidase (GPx) (10), suggesting that maspin might regulate cellular oxidative stress. Our laboratory had previously shown that in normal mammary epithelial MCF-10A cells, maspin forms intramolecular disulfide bond when exposed to a high level of oxidative stress [hydrogen peroxide (H₂O₂)], suggesting that maspin may regulate cellular ROS through its cysteine residues (11).

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 them 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 [-SO₂H] and sulfonic [-SO₃H] acid) (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, located at positions C183, C205, and C323 are structurally fully exposed. Given the anti-oxidant capacity of the cysteine thiol group, we hypothesized that these exposed cysteine residues in maspin may act as a potent scavenger/quencher of ROS. In the present 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.

EXPERIMENTAL PROCEDURES

**Plasmid constructs and cell culture**

Maspin X-ray crystal structure was 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 (PDB ID: 1xu8), as reported by Law et al (16) to analyze the presence of exposed cysteine residues, which can serve as site for oxidation. QuikChange II site-directed mutagenesis kit (Stratagene) was used to mutate cysteine residues to serine residues in pEF-IRE-Neo-h.maspin using the specific mutagenic primers (Table-S1). 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-IRE-Neo-h.maspin wild type (Mp), triple mutation (T) or control vector alone (Neo) by Effectent 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-h.maspin (GST-MpWT) and GST-h.maspin triple mutation (GST-MpT)] were induced by IPTG (1 mM), and purified using glutathione agarose (Sigma). Thrombin was used to cleave the maspin from the agarose beads. The size and purity of proteins were confirmed by SDS-PAGE and Western blot analysis.

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

**Western Blot Analysis**

Cell lysates were prepared in RIPA buffer with protease cocktail inhibitor (Thermo Scientific).
Cellular debris was cleared from lysates by centrifugation and protein concentration was determined by the BCA Protein Assay (Pierce). Samples were separated on 10% SDS-PAGE, transferred to a PVDF membrane (GE Healthcare) and blotted with rabbit anti-maspin AbS4A antibody (3) and anti-actin antibody (Cat No. A2066. Sigma). HRP labeled goat anti-rabbit polyclonal antibody was used as a secondary antibody and proteins were visualized with enhanced chemiluminescence substrate (Pierce).

**Quantification of Reactive Oxygen Species (ROS)** – Flurogenic substrate carboxy- 2’, 7’-dicholorodi hydrofluorescein diacetate (DCFH-DA) was used to detect intracellular ROS (22). Briefly, 10^6 cells in 6-well plate were plated and incubated at 37°C overnight. Next day, the culture medium was discarded and cells were washed twice with PBS followed by incubation with 20 μM Carboxy-DCFH-DA at 37°C for 30 minutes in serum free medium. Cells that were either untreated or treated with different ROS inducers at the indicated final concentrations were incubated at 37°C for indicated time periods. At the end of the exposure period, cell supernatants were discarded; cells were washed with PBS and harvested using Tryptsin/EDTA. Cells were transferred to FACS tubes and 20,000 events were analyzed using a BC Epics XL Analyzer with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Results were depicted as fold change in fluorescent intensity.

**Measurement of Superoxide (O_2^-) 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 hydroethidine (HE) a fluorogenic probe, which is widely used to detect superoxide levels (23). TM40D^Neo and TM40D^Mp cells were treated with staurosporine (STS; 1μM) for 3 hrs and incubated with 10μM HE for 1 hr. 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 minutes on ice. Cell suspension was centrifuged at 1000xg for 5 minutes 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 DPBS. Cell lysates were mixed with an equal amount of 0.2 M solution of HClO in MeOH, and left undisturbed for 2 hrs to allow protein precipitation. Samples were centrifuged for 30 minutes at 20,000g at 4°C and 100μl of supernatant was mixed with an equal amount of 1 M solution of potassium phosphate buffer (pH 2.6). After, centrifuging at 20,000g for 15 minutes at 4°C, sample were analyzed by HPLC-MS. Instrumental setup for the analysis of HE and its oxidation products by HPLC-MS is depicted in Table- S2. HPLC peak areas were normalized to protein concentration.

**Cell-extracellular matrix (ECM) adhesion assay** – Adhesion of mouse mammary TM40D^Neo cells to extracellular matrix matrigel (BD Biosciences) was used to determine the biological activity of recombinant proteins. Adhesion assay was performed as described by Corbett et al (26). Briefly, cells were suspended in medium containing 100 nM of maspin (GST.Mp^WT and GST.Mp^T) or GST, and then cultured on matrigel coated 96-well plate at a density of 5×10^4 cells/well in triplicates for 4 hrs. 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, 0.05 M citrate buffer, pH 5) for 1.5 hrs at 37°C. After incubation 75μl of development buffer (5 mM EDTA, 50 mM glycine, pH 10.4) was added and the 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 (Cat No. V6611, Promega).

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 WT (Mp\textsuperscript{WT}) and triple mutated (Mp\textsuperscript{T}) recombinant maspin proteins were oxidized with H\textsubscript{2}O\textsubscript{2} (1 mM) for 1 minute in the presence of dimedone (10 mM). The oxidation of cysteine residues was detected by Western blot analysis using an antibody (Millipore; Cat No. 07-2139) that specifically recognizes dimedone derivatized cysteine sulfenic acid residues.

Trapping of sulfenic acid in cells (Immunoprecipitation) – Cells were first treated with H\textsubscript{2}O\textsubscript{2} (250 µM) for 3 hrs and then lysed in lysis buffer: 100 mM Tris (pH 7.4), 1% Triton X-100, protease inhibitor mixture (Thermo Scientific) with or without dimedone (10 mM) for 45 minutes before the addition of N-ethylmaleimide to a final concentration of 100 mM for another 10 minutes. Cellular debris was cleared from lysates by centrifugation, and protein concentration was determined by the BCA Protein Assay (Pierce). Whole cell extracts (1 mg) were incubated overnight (constant rocking) with 0.5 µg of rabbit anti-maspin AbS4A antibody (3) or control rabbit IgG at 4°C. Protein A-Sepharose-coupled beads (Amersham Biosciences) were added and incubated for 1 hr at 4 °C under constant agitation. Beads were centrifuged, washed briefly with ice-cold lysis buffer, and finally incubated with elution buffer for 15 minutes at room temperature. Samples were mixed with non-reducing buffer (5X) and separated on SDS-PAGE gels, transferred to a PVDF membrane (GE Healthcare), and probed for maspin, cysteine sulfenic acid and IgG. Appropriate secondary antibodies were added, and proteins were visualized with enhanced chemiluminescence substrate (Pierce). Anti-Cysteine Sulfenic Acid was obtained from Millipore (Cat No. 07-2139), which recognizes proteins containing the dimedone-bound cysteine sulfenic acid.

Soft agar colony formation assay – As described previously (28), 6–well dishes were plated with bottom agar (0.7% agarose) for 30 minutes. Cells were mixed with the top agar (0.3% agarose) at a concentration of 5x10\textsuperscript{4} cells per well and allowed to solidify. The cells were fed every 5\textsuperscript{th} day with media and grown at 37°C for 3 weeks, cells stained for 1 hr with 0.05% crystal violet and the numbers of purple colored colonies were counted as 10 fields per well at 40X using Image J software with a cut off range of 20-5000 pixels.

MTT in vitro cell proliferation assay – Cells were seeded at 10\textsuperscript{3} cells/well in 96–well plate and allowed to grow at 37\degree\textsuperscript{C} with 5% CO\textsubscript{2}. At each time point MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide) reagent (5 mg/ml) was added in a volume of 10 µl per well and incubated at 37\degree\textsuperscript{C} with 5%CO\textsubscript{2} for 3 hrs. The media was aspirated and 100 µl of DMSO 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 (30µg) were separated on 12% SDS-PAGE and phospho-plus kit was used to determine the ERK1/2 phosphorylation (Cat No. 9100, Cell Signaling Technology).

Statistical Analysis – All the 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 primary mouse mammary epithelial cells isolated from wildtype (WT) and heterozygous maspin knockout (KO) mice and maspin silenced immortalized human mammary epithelial MCF-10A cells – In order to understand the role of maspin in oxidative stress, we isolated primary mammary
epithelial cells from WT and maspin heterozygous KO mice and measured their ROS levels. Fig. 1A depicts the Western blot analysis for maspin expression in cells isolated from WT and maspin heterozygous KO mice. We observed that the cells isolated from maspin KO mice had significantly higher intracellular ROS levels as compared with WT mice (p<0.001; Fig. 1B). We also observed a significant increase in ROS production in KO cells as compared with WT cells when they were pre-treated with H₂O₂ for 3 hrs (p<0.05; Fig. 1C). 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. 1D). MCF-10A cells displayed significantly lower levels of ROS as compared with the maspin-silenced cells MCF-10A229 cells (p<0.01; Fig. 1E). Collectively, these results indicate that presence of maspin in primary and in immortalized mammary epithelial cells make 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, TM40D mouse mammary epithelial cell line that expresses low levels of maspin, was used as a cellular model (Fig. 2A). Maspin was over expressed in TM40D cells (Fig. 2A) and total intracellular ROS levels were measured. Our results demonstrated that the exposure for 3 hrs to 250 µM H₂O₂ was sufficient to induce significant increase in intracellular ROS in TM40DNeo cells as compared to maspin overexpressing TM40Dmp cells (p<0.001; Fig. 2B). 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. 2C).

Additionally, hydroethidine (HE) was used to determine the superoxide (O₂⁻) levels as an indicator of oxidative stress in response to an alternative ROS inducer i.e. staurosporine (STS) using HPLC-MS in TM40DNeo and TM40Dmp cells. Figure 2D depicts the schematic representation of the reaction of HE and superoxide radical anion (O₂⁻) to form 2-hydroxyethidine (2-OH-E⁻). We observed a significant decrease in the levels of superoxide in maspin overexpressing cells as compared to TM40DNeo cells in response STS treatment (p<0.05; Fig. 2E).

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 presence of 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 reported by Law et al. (PDB ID: 1xu8) (16). We identified that out of eight cysteine residues present in human maspin only three residues at the position 183, 205, and 323 were structurally exposed, which might serve as possible sites for oxidation. In contrast, cysteine residues at position 20, 34, 214, 287, 373 were found to be buried. Previously, it has been shown that mutating all the cysteines to serine/alanine by site directed mutagenesis does not change the structure of maspin (17). Therefore, we mutated either single (C183 or C323) or all the three exposed cysteine residues to serine. Fig. 3A depicts three-dimensional structure of maspin demonstrating three structurally exposed (C183, C205 and C323) cysteine residues. TM40D cells were transfected with different maspin cysteine mutants and expression of maspin was confirmed by Western blot analysis (Fig. 3B).

Mutating a single cysteine residue i.e. 183 or 323 individually in maspin led to
a two-fold increase in ROS levels in comparison to TM40DMp cells (Fig. 3C); suggesting that both these cysteine residues are equally important in regulating cellular ROS. When all the three surface cysteines were mutated, cellular ROS level in TM40DT was further increased (~1.4 fold) compared with TM40D<sup>C183S</sup> or TM40D<sup>C323S</sup> cells (p<0.05; Fig. 3C). Increased ROS levels in TM40DT 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 glutathione redox cycle play an important role in detoxification of various oxidant species (31). A decreased GSH/GSSG ratio is considered indicative of increased oxidative stress (32,33). We found that overexpression of maspin in TM40D cells resulted in an increased ratio of GSH/GSSG (p<0.01; Fig. 3D). However, expressing triple mutant maspin in TM40D cells resulted in a decreased ratio of GSH/GSSG as compared with cells expressing WT maspin in the absence of ROS stimulus (p<0.01; Fig. 3D).

**Triple mutated maspin is biologically active and evidence for oxidation of cysteine thiol to sulfenic acid** – To confirm that mutated maspin retains its normal biological activity, we tested whether the triple mutated recombinant maspin (GST.Mp<sup>T</sup>) could increase cell adhesion to ECM matrix as similar to WT maspin protein (GST.Mp<sup>WT</sup>). Increased adhesion of TM40D<sup>Neo</sup> cells to matrigel matrix was observed when cells were pretreated with GST.Mp<sup>T</sup> compared to its control (p<0.01; Fig. 4A).

In order to elucidate whether selected cysteine residues of maspin traps free radicals or other oxidants, WT and triple mutated recombinant maspin proteins were treated with hydrogen peroxide 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 (15) (Fig. 4B). When probed with anti-cysteine sulfenic acid antibody, we observed an absence of signal in lanes loaded with triple mutated maspin as compared to WT maspin (Fig. 4C). 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 hydrogen peroxide treated TM40D<sup>Mp</sup>, TM40D<sup>C183S</sup>, TM40D<sup>C323S</sup> and TM40D<sup>T</sup> cells in the presence or absence of dimedone and the IP-products were analyzed for the presence of dimedone derivatized cysteine sulfenic acid residues under non-denaturing conditions. Figure 4D shows the presence of maspin cysteine sulfenic acid in TM40D<sup>Mp</sup>, TM40D<sup>C183S</sup> and TM40D<sup>C323S</sup> cells but not in the TM40D<sup>T</sup> cells. These results further demonstrate importance of structurally exposed cysteine residues. Similar results were obtained when the experiment was performed with human mammary cancer epithelial MCF-7 cells (Fig. 4D, bottom panel), which constitutively expresses 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 in maspin WT and mutant TM40D cells using soft agar colony formation and MTT assays respectively. Anchorage-independent growth is one of the hallmarks of transformation and 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. 5A. Compared with the vector transfected cells, maspin overexpressed TM40D<sup>Mp</sup> cells formed significantly fewer colonies/well (p<0.01; Fig. 5A), a feature indicative of
decrease in anchorage-independent growth, whereas no significant difference was observed when vector control was compared with the triple mutated maspin (p>0.05). A significant increase was observed in the colony formation between TM40D<sup>Mp</sup> and TM40D<sup>T</sup> cells (p<0.01). Results of MTT proliferation assay revealed that WT maspin overexpressing TM40D<sup>Mp</sup> cells have low proliferation rate when compared with TM40D<sup>Neo</sup> and TM40D<sup>T</sup> cells over a time period of 96 hrs (Fig. 5B). To obtain direct evidence that deviation in proliferation pattern in TM40D<sup>Neo</sup> and TM40D<sup>T</sup> cells is due to changed ROS levels, we treated both types of cells with N-acetyl cysteine (NAC; a strong antioxidant). We observed a significant drop in proliferation rate of TM40D<sup>Neo</sup> and TM40D<sup>T</sup> cells with the NAC treatment, whereas, no significant change was observed in TM40D<sup>Mp</sup> cells (Fig. 5C). 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 like ERK1/2 (38). The total ERK1/2 MAP kinase level was found to be nearly constant in all three cell lines derived from TM40D by Western blot analysis (Fig. 5D). However, the corresponding phosphorylated activated forms (p-ERK1/2) were found to be consistently increased in TM40D<sup>Neo</sup> and TM40D<sup>T</sup> cells as compared to TM40D<sup>Mp</sup> cells suggesting that TM40D<sup>Neo</sup> and TM40D<sup>T</sup> cells have higher constitutive expression of activated pERK1/2 than TM40D<sup>Mp</sup> cells (Fig. 5D). Treating cells with the anti-oxidant NAC significantly reduced the levels of activated pERK1/2 (Fig. 5E) and cell proliferation rate in TM40D<sup>Neo</sup> and TM40D<sup>T</sup> cells, however, no change was observed in TM40D<sup>Mp</sup>. These results, clearly demonstrate that maspin inhibits ERK1/2 activities, likely through ROS scavenging and this mechanism controls the epithelial cell proliferation.

DISCUSSION

Oxidative damage to any cellular constituent, if remained unchecked, can lead to disease development (1,39). Maspin belongs to the serpin family of non-inhibitory protease inhibitor (3) and is abundantly produced in normal mammary luminal epithelial and myoepithelial cells (3,40). Our laboratory and others have demonstrated maspin acts as a multifaceted protein, interacting with extracellular 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). Role of maspin in oxidative stress has been speculated in the last few years (9,11). In the present study, we provide evidence for oxidation of cysteine residues in maspin for the first time. Additionally, we demonstrate that maspin acts as a ROS scavenger to provide resistance against oxidative stress. This suggests a new paradigm that maspin allows tumor cells to proliferate in an environment of oxidative stress by maintaining redox homeostasis. Our data also shows that surface-exposed cysteines regulate ROS as an intracellular serpin. Results of adhesion assay (Fig. 4A) clearly demonstrate that this function of ROS regulation is independent of maspin’s extracellular function, which increases cell adhesion to ECM 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 MnSOD leads to an 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 condition, different oxidative agents may also modify maspin cysteines. We therefore
tested whether maspin expression level in normal mammary epithelial cells or in cells with 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. 2B) in maspin overexpressing mouse mammary epithelial cell (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. 2C) of maspin in oxidative stress, which is consistent with our finding that maspin attenuates ROS species such as superoxide (O$_2^-$) in STS-stressed TM40D cells (Fig. 2E). Taken together, our results indicate that presence of maspin in mammmary cells make them more efficient to fight against ROS irrespective of their origins; whether they are derived from mouse tumor epithelial cell (TM40D), or primary mouse epithelial cells, or human mammary epithelial MCF-10A cells.

Emerging evidence suggests that redox-sensitive cysteine residues in proteins may function as an oxidant sensor (44,45). The conversion of these residues to sulfenic acid has been demonstrated for redox signaling in yeast, T-cell activation, and in other proteins (14,15,45), providing strong support for the growing roles of this modification in biology. Mutating three cysteine residues in maspin resulted in significant increase in intracellular ROS levels in TM40D$^T$ cells (Fig. 3C). Further, levels of other anti-oxidant proteins were also found to alter in cells expressing cysteine to serine mutant maspin (TM40D$^T$) when compared with the WT maspin expressing cells (TM40D$^M$). Our data suggests that these cysteines have a cumulative effect because mutation of either C183S or C323S led to similar significant increase in cellular ROS level. The cells with triple mutations in maspin had ~1.4 fold increase in ROS level than cells with single cysteine mutation (Fig. 3C). However, there is still a possibility that certain redox agents may preferentially attack a particular cysteine on maspin in vivo depending on their size and structure. Future studies to determine whether certain redox agents affect different cysteines on maspin with different regulatory ability on 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, which forms more colonies (Fig. 5A). Results of MTT assay revealed that TM40D$^{Neo}$ cells proliferate more rapidly than WT maspin overexpressing cells. Increased proliferation of TM40D$^{Neo}$ cells coincides with the increased ROS levels in these cells (Fig. 2B and 5B). Cells with mutated maspin (TM40D$^T$) also proliferate more rapidly when compared to TM40D$^M$ cells (Fig. 5B). In agreement with this, Cia et al have also reported decreased proliferation rate with the maspin overexpression in esophageal carcinoma (46). Also, NAC treatment reversed the oxidative stress phenotype of the maspin deficient or mutated maspin cells and thus indicating proliferation was 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 (mitogen activated protein kinases) that are important in cell proliferation, differentiation, invasion, and apoptosis (47). In the present study, we found higher levels of phospho-ERK1/2 in TM40D$^{Neo}$ and TM40D$^T$ cells as compared with TM40D$^M$ cells (Fig. 5D). Anti-oxidant pre-treatment led to a decrease in expression of pERK1/2 in these cells (Fig. 5E). These observations suggest that an increased oxidative stress activates ERK1/2, which in turn increases proliferation. These results collectively substantiate that maspin overexpressing cells have lower ROS and therefore maintain lower levels of pERK1/2 and proliferation rates, as compared with cell lines that have attenuated or mutated
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) which 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 mutated maspin demonstrates the absence of above proposed mechanism. Cells expressing WT maspin have low proliferation rate, which is attributed to the 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 level.

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ACKNOWLEDGEMENTS. The work was supported by NIH CA79736 to M.Z. We thank Dr. Navdeep S Chandel at Northwestern University for helpful discussion. We also thank Dr. Xiaolin He for helping us identifying surface cysteines of maspin using Molsoft ICM-pro Version 3.48.

Author contributions: NM and MZ conceived the study, designed and interpreted the experiments, and wrote the paper. NM and HYS performed the experiments. TL suggested and helped in designing experiments related to dimedone and sulfenic acid and edited sections of the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.
FIGURE 1. Total intracellular ROS levels in primary mammary epithelial cells isolated from WT and heterozygous maspin KO mice and maspin silenced immortalized human mammary epithelial MCF-10A. Total intracellular ROS levels were measured by flow cytometric analysis using Carboxy-DCFH-DA 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 amount of proteins (25µg/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 level in primary mammary epithelial cells isolated from WT and heterozygous maspin KO mice (n=3) after treatment with H2O2 (250 µM) for 3 hrs. (D) Western blot analysis of maspin expression in immortalized human mammary epithelial MCF-10A and MCF-10A229 cells. (E) ROS level in immortalized human mammary epithelial MCF-10A and MCF-10A229 (maspin silenced) cells after treatment with H2O2 (250 µM) for 3 hrs. Data shown are mean±SD of three independent experiments. Asterisks indicate significance according to t-test (two -tailed); *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 TM40DNeo and TM40Dmp cells. Equal amount of proteins (25µg/lane) were loaded and immunoblotted for maspin and actin (a loading control). (B) ROS level in TM40DNeo and TM40Dmp cells after treating with H2O2 (250 µM) for 3 hrs. (C) ROS level in TM40DNeo and TM40Dmp cells after treating with antimycin-A (100 µM) for 1 hr. (D) Schematic representation of oxidation of hydroethidine (HE) and formation of various oxidation products. 2-hydroxyethidium (2-OH-E+) is the primary product of the reaction of HE and superoxide (O2°-) and other products like E+ and dimers of HE (HE-HE, HE-E+, E+E+) are indicators of one electron oxidants. (E) Levels of superoxide (2-OH-E+) in TM40DNeo and TM40Dmp cells after treating with STS (1 µM) for 3 hrs as measured by HPLC-MS. The results are shown as area under peak per mg of protein. Data shown are mean±SD of three independent experiments. Asterisks indicate significance according to t-test (two -tailed); *p<0.05, ***p<0.001.

FIGURE 3. Importance of cysteine residues and role in oxidative stress. (A) Three dimensional structure of maspin highlighting three cysteine residues marked in green were mutated to serine at position 183, 205 and 323. (B) Western blot analysis of maspin expression in TM40DNeo, TM40Dmp, TM40DT and other maspin mutant cells. Equal amount 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 H2O2 (250 µM) for 3 hrs as determined by flow cytometric analysis. (D) Constitutive levels of GSH/GSSG ratio in TM40DNeo, TM40Dmp and TM40DT cells. Data shown are mean±SD of three independent experiments. Asterisks indicate significance according to t-test (two -tailed); **p<0.01, ***p<0.001 compared with TM40Dmp; # p<0.05 compared with TM40DT.

FIGURE 4. Triple mutated maspin is biologically active and evidence for oxidation of cysteine to sulfenic acid. (A) Mutated maspin retains the ability to enhance cell adhesion to extracellular matrix (ECM). Increased adhesion of TM40DNeo cells to matrigel ECM was observed when cells were pretreated with wild type and triple mutated recombinant maspin (100 nM) as compared to its GST control. (B) Biochemistry of oxidation of thiol group [-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 sulfinic acid [-SO2H] and sulfonic acid [-SO3H]. Sulfenic acid can be labeled by dimedone or dimedone based
chemicals. (C) Equal amounts (62.5 ng) of WT and Triple mutated maspin were oxidized with H$_2$O$_2$ (10 mM) for 1 minute in presence of dimedone (10 mM) and oxidation of cysteine residues was detected by Western blot analysis using antibody, which specifically recognize dimedone derivatized cysteine sulfenic acid residues (D) Cells were treated with H$_2$O$_2$ (250 µM) for 3 hrs and then lysed in presence or absence of Dimedone (10 mM). Maspin was immunoprecipitated and then immunobloted with maspin antibody and antibody, which specifically recognizes dimedone derivatized cysteine sulfenic acid residues. Data shown are mean±SD of three independent experiments. Asterisks indicate significance according to t-test (two -tailed); **p<0.01, ***p<0.001.

FIGURE 5. Cysteine mutated cells have increased cellular proliferation and ERK1/2 activation. (A) Number of colonies formed in vitro using a soft agar colony formation assay by different cell lines. (B) Comparison of cellular proliferation pattern as determined by MTT assay in three different cell lines. (C) Treatment with anti-oxidant (i.e. NAC) significantly decreases the proliferation in TM40D$_{Neo}$ and TM40D$_T$ cells. (D) Comparison of pERK1/2 levels in TM40D$_{Neo}$ and TM40D$_T$ cells compared with TM40D$_{Mpo}$ cells. (E) Effect of NAC treatment on pERK1/2 levels in TM40D$_{Neo}$, TM40D$_{Mpo}$ and TM40D$_T$ cell. Representative Western blots are shown above the bar diagrams and each lane corresponds to respective bars. Data shown are mean±SD of three independent experiments. Asterisks indicate significance according to t-test (two -tailed); **p<0.01, ***p<0.001.
Fig. 4

A

![Graph showing OD405 values for GST, GST-Mp WT, and GST-Mp T](image)

B

![Chemical reactions involving thiol, sulfinic acid, and dimedone derivatized cysteine sulfenic acid](image)

C

![Western Blot images for WB, rMp WT, and rMp T](image)

D

![Western Blot images for different samples and treatments](image)