FORUM ORIGINAL RESEARCH COMMUNICATION

Manganese Superoxide Dismutase Expression Regulates the Switch Between an Epithelial and a Mesenchymal-Like Phenotype in Breast Carcinoma

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Abstract

Aim: Epithelial–mesenchymal transition (EMT) is characterized by the acquisition of invasive fibroblast-like morphology by epithelial cells that are highly polarized. EMT is recognized as a crucial mechanism in cancer progression and metastasis. In this study, we sought to assess the involvement of manganese superoxide dismutase (*MnSOD*) during the switch between epithelial-like and mesenchymal-like phenotypes in breast carcinoma.

Results: Analysis of breast carcinomas from The Cancer Genome Atlas database revealed strong positive correlation between tumors' EMT score and the expression of *MnSOD*. This positive correlation between *MnSOD* and EMT score was significant and consistent across all breast cancer subtypes. Similarly, a positive correlation of EMT score and *MnSOD* expression was observed in established cell lines derived from breast cancers exhibiting phenotypes ranging from the most epithelial to the most mesenchymal. Interestingly, using phenotypically distinct breast cancer cell lines, we provide evidence that constitutively high or induced expression of *MnSOD* promotes the EMT-like phenotype by way of a redox milieu predominantly driven by hydrogen peroxide (H_2O_2). Conversely, gene knockdown of *MnSOD* results in the reversal of EMT to a mesenchymal–epithelial transition (MET)-like program, which appears to be a function of superoxide ($\text{O}_2^{\bullet-}$)-directed signaling. **Innovation and Conclusion:** These data underscore the involvement of *MnSOD* in regulating the switch between the EMT and MET-associated phenotype by influencing cellular redox environment *via* its effect on the intracellular ratio between $\text{O}_2^{\bullet-}$ and H_2O_2 . Strategies to manipulate *MnSOD* expression and/or the cellular redox milieu *vis-a-vis* $\text{O}_2^{\bullet-}$: H_2O_2 could have potential therapeutic implications. *Antioxid. Redox Signal.* 25, 283–299.

Introduction

EPITHELIAL–MESENCHYMAL TRANSITION (EMT) is the process where epithelial cells undergo changes that enhance their migratory ability, invasiveness, expression of

extracellular matrix (ECM) proteins, and resistance to stress- and/or death-inducing stimuli (39, 47). The phenotypic and functional attributes are associated with a switch in the expression of biological markers that are characteristic of epithelial cells such as E-cadherin to those that dictate the

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Innovation

Overexpression of manganese superoxide dismutase (MnSOD) is observed in mesenchymal-like breast cancers that exhibit increased migratory, invasive, and metastatic capacities. MnSOD appears to drive epithelial–mesenchymal transition (EMT) *via* its ability to modulate cellular redox environment *via-a-vis* the ratio of superoxide: hydrogen peroxide. Not only do these data provide a novel insight into the cross talk between cellular redox milieu and the acquisition of EMT but more importantly also have potential implications for designing specific therapeutic strategies to exploit the vulnerabilities of aggressive and refractory breast cancers.

acquisition of the mesenchymal morphology such as N-cadherin, vimentin, and fibronectin (15). EMT plays a critical role in the progression of carcinogenesis from a localized (*in situ*) to an invasive (metastatic) disease. The reverse process, mesenchymal–epithelial transition (MET), is involved in the conversion of micrometastasis into macrometastasis, thus forming secondary tumor sites (44).

Luminal breast cancer cell lines such as MCF7 and T47D have been associated with low invasiveness, while basal-like breast cancer cell lines such as MDA-MB-231 and BT549 are highly metastatic and invasive (4). The progression from noninvasive to invasive tumors has also been proposed to involve EMT. In breast carcinoma, basal-like cancers, which have a highly invasive phenotype, express high levels of mesenchymal markers (Fibronectin, N-cadherin, α -smooth muscle actin) and ECM remodeling proteins (laminin, fascin) and, correspondingly, reduced expression of E-cadherin and cytokeratin, proteins associated with the epithelial phenotype. Thus, since basal-like tumors are often more aggressive and invasive, this strengthens the association between EMT and cancer progression (40).

A 68% correlation in advanced breast cancer patients was reported between increased Twist and V-Akt murine thymoma viral oncogene homolog 2 compared with 13% in early stage breast cancer (9). Blanco *et al.* also analyzed primary tissue derived from patients with breast cancer and reported a positive correlation between Snail expression and metastatic potential (3). Relapse-free survival and risk of relapse were also lower in women with high Snail levels, demonstrating that Snail could be a potential clinical prognostic indicator (33). Therefore, finding ways to inhibit or reverse EMT could be a favorable approach against basal-like breast cancer.

EMT is tightly regulated by various processes: induction of transcription factors (TFs), *de novo* expression of cytoskeletal and surface proteins, restructuring of cytoskeleton assembly, alterations in integrin expression, induction of ECM-degrading enzymes such as matrix metalloproteinases (MMPs), cathepsins, urokinase-type plasminogen activator protease, and activation of specific microRNAs (17, 18). Hypoxia-inducible factors are also strong inducers of EMT-linked TFs, such as Snail, Slug, zinc finger E-Box binding homeobox 1 (Zeb1), and zinc finger E-Box binding homeobox 2 (Zeb2), coupled with genes involved in metastasis, such as vascular endothelial growth factor, cathepsin D, and MMP2 (21, 45).

Manganese superoxide dismutase (MnSOD) is an important antioxidant enzyme located in the mitochondrial matrix

that specifically promotes the conversion of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), which is later decomposed to oxygen and H_2O by glutathione peroxidase and catalase (19). The human MnSOD protein, an 88 kDa homotetrametric enzyme, is the product of a nuclear-encoded gene mapped to chromosome 6q25. The 5'-proximal promoter in the *MnSOD* gene is characterized by an enrichment of GC boxes, with enhancer elements in the second intron (23, 50). There are various TF binding motifs within the promoter and enhancer regions of *MnSOD* that regulate its transcription. Factors that regulate *MnSOD* transcription include nuclear factor-kappa B, specificity protein 1 (Sp1), activating protein-1, p53, CCAAT-binding protein (C/EBP), nuclear factor (erythroid-derived 2)-like 2, and Forkhead box O1 (11).

Our recent findings and that of others demonstrated significantly higher MnSOD levels in the highly invasive BT-549 and MDA-MB231 cell lines (triple negative breast cancer [TNBC]) compared with the noninvasive (MCF-7 and T47D) or nontumorigenic cell lines (MCF-12A and MCF-12F) (25, 26, 34). Interestingly, recent research has unraveled possible causes for the high variability in *MnSOD* expression in cancers. The authors demonstrate a decrease in the expression of *MnSOD* *in vivo* during the initial stage of carcinogenesis, thereby corroborating its role as a tumor suppressor. However, *MnSOD* expression increases when cells acquire a more aggressive and invasive phenotype, conferring protection against mitochondrial injury and oxidative stress, which is commonly seen in triple negative breast tumors (11–14). MnSOD-dependent upregulation of Forkhead Box M1 (FoxM1) transcriptional activity could also drive the expression of MMP2, a key player in stromal degradation associated with tumor invasion (8). Overexpression of MnSOD in the MCF7 breast tumor cell line has also been demonstrated by Oberley and colleagues to stimulate the activation of MMP2 (51). In addition, upregulated MnSOD in breast cancer is associated with anoikis resistance and metastasis (24). These data provide testimony that overexpression of MnSOD contributes to the acquisition of the EMT phenotype.

Notably, Meng *et al.* reported that RNAi-mediated suppression of miRNA-212 repressed *MnSOD* expression with a corresponding reduction in mesenchymal markers—in line with inhibition of the EMT program—in colorectal cancer cells (31). Conversely, exposure of MCF7 cells with estradiol or 27-hydroxycholesterol upregulated MnSOD levels and promoted EMT (10). Taken together, these findings reveal a critical role of MnSOD as a potential regulator of EMT in breast carcinoma and therefore a potential target for therapeutic intervention.

Previously, our work underscored the potential therapeutic benefit of targeting MnSOD for reactive oxygen species (ROS)-dependent execution of aggressive basal-like breast tumors (26). Since EMT imparts invasiveness and metastatic potential to breast tumors and MnSOD reveals itself to bear potential association with EMT, we aim to investigate the contribution of MnSOD in controlling the switch between epithelial and mesenchymal phenotypes in breast carcinoma. Using an EMT score analysis, we show that MnSOD gene expression is significantly and positively correlated with EMT in all subtypes of breast cancer patients. Moreover, we demonstrate how overexpression of MnSOD in a cell line model of a basal-like/claudin-low and luminal breast

FIG. 1. EMT score and MnSOD expression. (A) Dot plot showing MnSOD expression levels (x-axis) and EMT score (y-axis) in all breast cancer subtypes ($n=536$). (B) Dot plot showing MnSOD expression levels (x-axis) and EMT score (y-axis) in basal and claudin-low subtypes ($n=98$). (C) Dot plot showing MnSOD expression levels (x-axis) and EMT score (y-axis) in luminal subtype ($n=351$). (D) Dot plot showing MnSOD expression levels (x-axis) and EMT score (y-axis) in ERBB2+ subtype ($n=51$). Correlation ρ and p -values computed by Spearman's correlation coefficient test are shown in graphs (A–D). (E) Bar plots of EMT score of breast cancer cell lines aligned from the most epithelial (EMT score = -1.0) to the most mesenchymal (EMT score = +1.0) and of the corresponding MnSOD gene expression (F). Names of cell lines are labeled on the x-axis. Gray-colored bars indicate cell lines used in the article. EMT, epithelial–mesenchymal transition; MnSOD, manganese superoxide dismutase.

carcinoma favored the formation of a mesenchymal phenotype with an increase in markers of EMT and cells' scattering, invasive, and motile capacity. On the other hand, repression of MnSOD induces an epithelial phenotype with a reduction in EMT markers and cells' scattering, invasive, and motile capacity. Furthermore, regulation of the epithelial *versus* mesenchymal-like phenotype by MnSOD was shown to be associated with different cellular redox milieu with respect to the $O_2^{\cdot-}:H_2O_2$ ratio.

Results

Positive correlation between MnSOD expression and the EMT score of human breast cancer tumors and breast cancer cell line models

Primary tissue obtained from breast cancer patients ($n=536$) from Agilent custom gene expression microarray G4502A_07 platform was scored for EMT and *MnSOD* expression. Interestingly, *MnSOD* expression is significantly and positively correlated with the tumors' EMT scores when analyzed in a breast cancer subtype-independent manner (Fig. 1A; $\rho=+0.6308$, $p<1.0E-359$). Interestingly, the positive correlation of MnSOD and EMT is significantly and consistently maintained across all breast cancer subtypes: basal and claudin low (Fig. 1B; $\rho=+0.4066$, $p=3.8E-5$), luminal (Fig. 1C; $\rho=+0.4544$, $p<1.0E-359$), and ERBB2+ (Fig. 1D; $\rho=+0.6213$, $p=2.1E-6$). Of note, corresponding to their epithelial phenotype (Fig. 1C), the luminal subtype of breast cancer maintains an EMT score smaller than zero. Conversely, the basal and claudin-low subtypes maintain an EMT score greater than zero, which is in agreement with their mesenchymal phenotype (Fig. 1B). Interestingly, similar to the data obtained upon analysis of patient-derived primary tissue, the EMT score and MnSOD expression in a series of breast cancer cell lines showed that cell lines representative of Basal-like/claudin-low breast cancer tumors, such as MDA-MB231 and BT549, showed higher MnSOD expression and EMT score compared with cell lines associated with luminal breast tumors, such as MCF-7 (Fig. 1E, F).

Downregulation of MnSOD expression favors a switch from an EMT-like phenotype to an MET-like phenotype

Intrigued by the strong positive correlation between EMT score and MnSOD expression in clinical biopsies as well as in established breast cancer cell lines, we investigated the direct involvement of MnSOD in the regulation of the EMT-like *versus* MET-like phenotype. To do so, we selected the triple negative basal-like mesenchymal cancer cell lines (MDA-MB-231 and BT549) that express high levels of MnSOD to investigate the effect of decreasing MnSOD expression on the EMT-like phenotype. Knockdown of *MnSOD* in MDA-MB-231 cells was associated with a significant downregulation in the mRNA (Table 1) and protein expression of EMT markers such as Vimentin, N-Cadherin, active β -catenin (wingless-type MMTV integration site family [Wnt] signaling mediator), and EMT-associated TFs (49) *Slug*, *Twist*, *Zeb 2*, and *Snail* (Fig. 2A–D). Similar effect of *siMnSOD* on *N-cadherin*, *Snail*, and active β -catenin and conversely an increase in *E-cadherin* expression was observed in BT549 cells (Supplementary Fig. S1A–D;

TABLE 1. RELATIVE GENE EXPRESSION CHANGES OF EPITHELIAL–MESENCHYMAL TRANSITION-ASSOCIATED MARKERS AND EPITHELIAL–MESENCHYMAL TRANSITION-ASSOCIATED TRANSCRIPTION FACTORS FOLLOWING DEPLETION OF MANGANESE SUPEROXIDE DISMUTASE EXPRESSION IN MDA-MB-231

<i>Fold change from ctrl siRNA $\pm$ SD (n=3)</i>	
EMT-associated markers	
Vimentin	0.50 ± 0.02
Fibronectin	0.24 ± 0.09
N-Cadherin	0.20 ± 0.04
EMT-associated transcription factors	
Snail	0.60 ± 0.05
Twist	0.32 ± 0.09
Zeb1	0.61 ± 0.04
Zeb2	0.29 ± 0.12

MDA-MB-231 cells were transfected with control scrambled RNA (ctsi) or 100 nM *siMnSOD*. Forty-eight hours post-transfection, cells were harvested and analyzed by RT-PCR for relative mRNA levels of the EMT-associated genes. Gene expression was normalized to GAPDH mRNA levels. Results are expressed as fold change against ctsi. Results shown represent mean $\pm$ SD, $n=3$.

EMT, epithelial–mesenchymal transition; MnSOD, manganese superoxide dismutase; RT-PCR, real-time polymerase chain reaction; SD, standard deviation; Zeb1, zinc finger E-Box binding homeobox 1; Zeb2, zinc finger E-Box binding homeobox 2.

Supplementary Data are available online at www.liebertpub.com/ars.

To assess whether the effect observed on EMT markers upon *MnSOD* knockdown impacted functional attributes linked to the EMT phenotype, the scattering ability and migratory and invasive capacities of MDA-MB-231 and BT549 cells were evaluated. First, the percentage distribution of scattered colonies significantly decreased in cells upon knockdown of *MnSOD* (Fig. 3A, B and Supplementary Fig. S2A, B), thereby indicating a shift toward an increased tendency to grow in tight colonies compared with highly scattered colonies in control cells. Morphologically, the normal fibroblast-like and elongated phenotype of MDA-MB-231 and BT549 cells was replaced by a more epithelial-like and polygonal appearance in response to MnSOD repression, thus suggesting a change from the mesenchymal-like appearance to a more epithelial-like phenotype through MET. Based on the effect of MnSOD repression on markers of EMT as well as the ability of cells to scatter, we hypothesized that a reversion to MET would cripple the ability of cells to migrate and/or invade. Corroborating that (using a wound healing assay) we show that *MnSOD* knockdown significantly reduced migratory ability as well as invasive capacity of MDA-MB-231 and BT549 cells (Fig. 3C–F and Supplementary Fig. S2C–F).

Increase in MnSOD expression favors a switch from an MET-like to EMT-like phenotype

Stimulated by our findings on the effect of knockdown of *MnSOD* in mesenchymal-like TNBC cell lines and our observed clinical data from patients' specimens, which clearly show an increase in the EMT score of the luminal subtype of breast tumors with high expression of MnSOD (Fig. 1C), we reasoned that the enforced expression of MnSOD in an

FIG. 2. Decrease in MnSOD expression induces the down-regulation of EMT-related protein expression in MDA-MB-231 breast cancer cell line. MDA-MB-231 cells were transfected with control scrambled RNA (ctsi) or 100 nM *siMnSOD*. Forty-eight hours post-transfection, cells were harvested and analyzed by Western blot for (A) Vimentin, (B) N-Cadherin, (C) β -catenin (active), and Slug, (D) Twist, Zeb2, and Snail. Knockdown efficiency on MnSOD expression is also shown for each figure. β -Actin was used as an endogenous control.

epithelial-like breast cancer cell line ought to favor the acquisition of an EMT-like phenotype. As expected, overexpression of MnSOD in MCF7 cells upregulated the expression of mesenchymal markers N-Cadherin, Vimentin, active β -catenin, and the EMT-associated TFs Snail, Twist, Zeb2, and Slug, while decreasing the MET marker E-cadherin assessed at the transcription and protein levels (Table 2; Fig. 4A–G). Similar changes in EMT-associated genes and TFs upon upregulating MnSOD were observed in two other epithelial-like breast cancer cell lines, T47D and BT474 (Supplementary Tables S1 and S2). Immunofluorescence further confirmed the positive correlation between MnSOD overexpression and induction of EMT markers, N-cadherin, active β -catenin, and Vimentin, as well as the negative effect on the MET marker E-cadherin (Fig. 4H–K). Similar changes to EMT-associated protein levels were observed in T47D and BT474 (Supplementary Figs. S3A–C and S4A–C). Furthermore, MnSOD overexpression resulted in a shift from the uniformly shaped polygonal morphology in MCF7 cells to a pronounced irregularly shaped, elongated, and fibroblast-like phenotype. Of note, cell-to-cell distance was greatly increased with minimal cell contact points and the number of scattered colonies was significantly higher in MCF cells overexpressing MnSOD (Fig. 5A, B). Importantly, overexpression of MnSOD in MCF7 cells induced a significant increase in the migratory capacity of cells toward the wound and invaded through the invasion chamber (Fig. 5C–F), thus indicating the acquisition of an EMT-like phenotype. Similar results in migration properties following MnSOD overexpression were shown using two other epithelial-like cell lines, T47D and BT474 (Supplementary Figs. S3D, E and S4D, E).

MDA-MB-231 DOX-R cells exhibit enhanced EMT-like functional attributes

Interestingly, a dramatic upregulation of MnSOD was observed in MDA-MB-231 breast cancer cells that had been

rendered resistant to the commonly used chemotherapeutic agent, doxorubicin (DOX) (MDA-MB-231 DOX-R). Having validated that ectopic overexpression of MnSOD promoted EMT in MCF7 breast tumor cells, the constitutively increased expression of MnSOD in MDA-MB-231 DOX-R cells (26) provided an excellent opportunity to validate the relationship between MnSOD and EMT. Indeed, the mRNA and protein expression of EMT-associated proteins, Vimentin, N-Cadherin, active β -catenin, Snail, and Slug were significantly increased in MDA-MB-231 DOX-R cells compared with the parental MDA-MB-231 cell line (Table 3; Fig. 6A–E). Not only did the MDA-MB-231 DOX-R cells exhibit an increase in EMT-related proteins but also elicited higher migratory and invasive capacities compared with the parental cell line (Fig. 6F–I). The latter is in agreement with the higher EMT score of clinical tumors within the basal and claudin-low subtypes that had higher expression of MnSOD (Fig. 1B).

MnSOD-induced EMT is a function of its redox modulating activity

Thus far, our results demonstrate that a high MnSOD expression is tightly associated with the acquisition of the EMT phenotype and functional hallmarks. Since MnSOD is a major antioxidant protein that serves to maintain the tight balance between intracellular $O_2^{\bullet-}$ and H_2O_2 , it is only logical to ask whether the effect of MnSOD is mediated through its redox modulating activity. In this regard, we previously reported that downregulation of MnSOD results in the accumulation of $O_2^{\bullet-}$, which can react with nitric oxide (NO) to generate peroxynitrite (OONO), while overexpression of MnSOD increases intracellular H_2O_2 (26). It is noteworthy that OONO[−] and H_2O_2 have been shown to regulate EMT (18, 36). In agreement with that *a priori* treatment of cells with the OONO[−] decomposition catalyst and scavenger, 5,10,15,20-Tetrakis(4-sulfonatophenyl) porphyrinato iron (III) chloride (FeTPPS), blocked the repressive effect of

FIG. 3. Decrease in MnSOD expression induces increased scattering, motile, and invasive capacity in the MDA-MB-231 breast cancer cell line. MDA-MB-231 cells were transfected with control scrambled RNA (ctsi) or 100 nM siMnSOD. **(A)** Cell scattering assay of cells after MnSOD repression. Forty-eight hours post-transfection, 1000 cells were reseeded into six-well plates and grown for 2 weeks. Colonies formed after 14 days were classified into three groups (tight, loose, and scattered) based on intercellular distance between cells in a colony. Representative images ($\times 10$ and $\times 20$ magnification) are shown. **(B)** *Top*: Western blot to show efficient knockdown of MnSOD after 14 days. *Bottom*: Numbers of colonies from **(A)** were counted and expressed as percentages of the total number of colonies formed. Results shown represent mean $\pm$ SD, $n = 3$. **(C)** Migration assay upon MnSOD suppression. Forty-eight hours post-transfection, cells were reseeded in 60-mm dishes and grown to 90% confluence. A wound was made and images were taken at 0, 24, 48, and 72 h to depict wound healing ability. **(D)** *Top*: Western blot to show efficient knockdown of MnSOD. *Bottom*: The wound length between cells in **(C)** was measured at three separate regions. Results shown represent mean $\pm$ SD, $n = 3$. **(E)** Invasion assay upon MnSOD suppression. Forty-eight hours post-transfection, cells were reseeded in into the upper chamber of a transwell invasion chamber with serum-free media and grown for 48 h. The membranes of the chambers were then stained with crystal violet solution and mounted on glass slides. Representative light microscopy images ($\times 100$) are shown. **(F)** *Top*: Western blot analysis to show efficient knockdown of MnSOD. *Bottom*: The number of cells from **(E)** that invaded through the transwell invasion chambers was counted in five randomly selected microscope fields. Results shown represent mean $\pm$ SD, $n = 3$. SD, standard deviation.

TABLE 2. RELATIVE GENE EXPRESSION LEVELS OF EPIHELIAL-MESENCHYMAL TRANSITION-ASSOCIATED GENES FOLLOWING ECTOPIC OVEREXPRESSION OF MANGANESE SUPEROXIDE DISMUTASE IN EPIHELIAL MCF-7 CELLS

Fold change from pcDNA3 ± SD (n = 3)	
EMT-associated markers	
E-Cadherin	0.35 ± 0.13
Fibronectin	3.20 ± 0.48
N-Cadherin	4.10 ± 0.50
β-Catenin	1.41 ± 0.05
EMT-associated transcription factors	
Snail	3.14 ± 0.60
Twist	1.16 ± 0.04
Zeb1	4.41 ± 0.59
Zeb2	2.13 ± 0.36

MCF7 cells were transfected with pcDNA3 vector or pcDNA3-MnSOD plasmid. Forty-eight hours post-transfection, cells were harvested and analyzed by RT-PCR for relative mRNA level changes of EMT-related genes normalized to GAPDH mRNA levels. Results are expressed as fold change against pcDNA3. Results shown represent mean ± SD, $n = 3$.

MnSOD silencing on the mRNA levels of *N-Cadherin*, *Vimentin*, and *Snail* in MDA-MB231 cells (Table 4). A decrease in the protein levels of β -catenin, Vimentin, Snail, and Slug upon MnSOD silencing was also blocked upon incubated with FeTPPS (Fig. 7A, B). Incubation with FeTPPS in MDA-MB-231 cells increased their invasion and migration properties (Fig. 7C–E), implying that ONOO[−] is a key player in driving the MET program. Furthermore, migration properties of MDA-MB-231 cells upon MnSOD silencing were restored upon incubation with FeTPPS (Fig. 7D, E), providing evidence that ONOO[−] is involved in MET induced by MnSOD knockdown. Conversely, preincubation of MCF7 cells with the ROS scavenger, *N*-acetyl cysteine (NAC), inhibited the EMT-promoting effect of MnSOD overexpression; decrease in *E-Cadherin* and a reciprocal increase in *N-Cadherin* and *Fibronectin* mRNA levels (Table 5). These data indicate that the EMT-promoting activity of *MnSOD* is linked to its ability to alter the intracellular redox milieu, in particular an imbalance in the O₂^{•−}:H₂O₂ ratio in favor of H₂O₂, while the MET-promoting effect is associated with an imbalance of the O₂^{•−}:H₂O₂ ratio in favor of O₂^{•−}, leading to the formation of ONOO[−].

Discussion

MnSOD is associated with promotion of EMT in breast cancer

In this study, we present evidence that MnSOD expression correlates positively with breast carcinoma EMT score. Breast cancer subtypes with mesenchymal hallmarks, such as the basal-like and claudin-low subtypes, express high levels of MnSOD. This observation was corroborated using a panel of breast cancer cell lines; cell lines that elicit mesenchymal-type morphology and the functional characteristics have significantly higher expression of *MnSOD* compared with cell lines that are more epithelial-like. The association between MnSOD expression and acquisition of the mesenchymal characteristics is corroborated by experiments using

human breast cancer cell lines with mesenchymal (MDA-MB-231 and BT549) *versus* epithelial phenotype (MCF7). While knockdown of *MnSOD* in the highly metastatic MDA-MB-231 and BT549 cells inhibited the expression of EMT markers as well as tumor cell invasive and migratory capacities, the converse was observed upon the enforced overexpression of MnSOD in the epithelial-like MCF7 cells. A further evidence to link MnSOD with the EMT program is provided in cells rendered resistant to the chemotherapeutic agent, DOX. Drug-resistant cells exhibited greater mesenchymal characteristics as well as a significant upregulation of MnSOD, and knockdown of *MnSOD* resulted in the switching off of the EMT program.

Knockdown of *MnSOD* in breast cancer cell lines harboring a mesenchymal phenotype (MDA-MB-231 and BT549) resulted in a significant downregulation in the expression of mesenchymal markers as well as TFs that mediate the repression of epithelial marker *E-Cadherin*, such as Snail, Slug, Twist, Zeb1, and Zeb2. Snail was shown to inhibit *E-Cadherin* expression by interacting with the proximal E-boxes on the promoter of *E-Cadherin* (7). Slug is able to form complexes with known corepressors of transcription NCoR and C terminal-binding protein 1 at the *E-Cadherin* promoter, thus downregulating its expression (32). Zeb1 was found to recruit histone deacetylases, HDAC1 and HDAC2, to the *E-Cadherin* promoter, repressing its expression (41). In addition to its ability to transcriptionally repress *E-Cadherin* expression, Zeb2 was reported to activate integrin $\alpha 5$ and Vimentin transcription *via* Sp1 recruitment (35). In addition, protein levels of active β -catenin, involved in forming adherent junctions between cells and a key player in the Wnt signaling pathway to promote EMT, were also lowered. Of note, active β -catenin has been shown to form a complex with Snail *via* extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, which activates Wnt and drives EMT progression (53).

Phenotypically, knockdown of *MnSOD* in aggressive basal-like breast cancer cell lines, MDA-MB-231 and BT549 cells, led to a marked change from the mesenchymal-like to epithelial-like morphology as well as inhibition of invasive and migratory capacities. Several studies across different cancer types have shown that MnSOD is a regulator of tumor migration and invasion. Chen *et al.* reported that the upregulation of FoxM1-MMP2 axis by MnSOD promotes lung tumor invasion (8). Another study showed that overexpression of MnSOD in glioma cells resulted in elevated MMP1 and MMP9 levels, contributing to tumor migration and invasion (27). Furthermore, stimulation of MnSOD expression by progesterin led to the activation of the mitogen-activated protein kinase (MAPK) pathway and increased migration and invasion of breast cancer cells (20). These studies corroborate our findings implicating high levels of MnSOD with acquisition of the EMT program and, as such, underscore the critical role of this mitochondrial protein in promoting tumor migration, invasion, and metastasis.

These findings correlate with previous reports demonstrating increased tumor invasion upon MnSOD overexpression in breast and other tumor cell types (8, 20, 27). An earlier report also highlighted the role of MnSOD in promoting invasiveness in breast cancer cells *via* upregulation of MMP-2 activity, required for cell migration and remodeling

FIG. 4. Enforced expression of MnSOD in epithelial MCF-7 (low basal MnSOD) cells induces and increases expression of EMT-related proteins. MCF7 cells were transfected with pcDNA3 vector or pcDNA3-MnSOD plasmid. Forty-eight hours post-transfection, cells were harvested and analyzed by Western blot for (A) E-Cadherin, (B) Vimentin (C) Slug and N-Cadherin (D) β -catenin, (E) Snail, (F) Twist, and (G) Zeb2. Increased MnSOD expression following transfection of pcDNA3-MnSOD plasmid is also shown in the above individual figures. β -Actin was used as an endogenous control. Forty-eight hours post-transfection, cells were reseeded on glass slides in 24-well plates and fixed for immunofluorescence staining. (H) Red E-Cadherin staining and blue DAPI staining were viewed under a confocal microscope. (I) Red N-Cadherin staining and blue DAPI staining were viewed under a confocal microscope. (J) Red β -catenin staining and blue DAPI staining were viewed under a confocal microscope. (K) Red Vimentin staining and blue DAPI staining were viewed under a confocal microscope. Images were captured at $\times 20$ magnification using an Olympus FluoView1000 microscope and are representative of three independent experiments. DAPI, 4',6-diamidino-2-phenylindole.

FIG. 5. Enforced expression of the MnSOD gene induces an EMT-like phenotype in epithelial MCF-7 (low basal MnSOD) cells with increased capacity to migrate and invade. MCF7 cells were transfected with pcDNA3 vector or pcDNA3-MnSOD plasmid. (A) Cell scattering assay upon MnSOD overexpression. Forty-eight hours post-transfection, 1000 cells were reseeded into six-well plates and grown for 2 weeks. Colonies formed after 14 days were classified into three groups (tight, loose, and scattered) based on intercellular distance between cells in a colony. Representative images ($\times 10$ and $\times 20$ magnification) are shown. (B) *Top*: Western blot to show efficient overexpression of MnSOD after 14 days. *Bottom*: Numbers of colonies from (A) were counted and expressed as percentages of the total number of colonies formed. Results shown represent mean $\pm$ SD, $n=3$. (C) Cell migration assay upon MnSOD overexpression. Forty-eight hours post-transfection, cells were reseeded in 60-mm dishes and grown to 90% confluence. A wound was made and images were taken at 0, 24, and 48 h to depict wound healing ability. (D) *Top*: Western blot to show efficient overexpression of MnSOD. *Bottom*: The wound length between cells was measured at three separate regions. Results shown represent mean $\pm$ SD, $n=3$. (E) Invasion assay upon MnSOD overexpression in MCF7 breast cancer cells. Seventy-two hours post-transfection, cells were reseeded into the upper chamber of a transwell invasion chamber with serum-free media and grown for 72 h. The membranes of the chambers were then stained with crystal violet solution and mounted on glass slides. Representative light microscopy images ($\times 10$) are shown. (F) The number of cells that invaded through the transwell invasion chambers from (E) was counted in five randomly selected microscope fields. Results shown represent mean $\pm$ SD, $n=3$.

TABLE 3. EXPRESSION OF EPITHELIAL–MESENCHYMAL TRANSITION-ASSOCIATED MARKERS AND EPITHELIAL–MESENCHYMAL TRANSITION-ASSOCIATED TRANSCRIPTION FACTORS IN MBA-MB-231-DOX-R COMPARED WITH THE PARENTAL MDA-MB-231 CELL LINE

	<i>Fold change from parental $\pm$ SD (n=3)</i>
EMT-associated markers	
Vimentin	2.41 $\pm$ 0.26
Fibronectin	2.81 $\pm$ 0.23
N-Cadherin	4.27 $\pm$ 0.30
β -Catenin	1.82 $\pm$ 0.21
EMT-associated transcription factors	
Snail	0.79 $\pm$ 0.34
Twist	3.18 $\pm$ 0.20
Zeb1	3.42 $\pm$ 0.30
Zeb2	2.85 $\pm$ 0.10

Real-time PCR of EMT-related genes in parental MDA-MB-231 compared with its doxorubicin-resistant (DOX-R) cells. Relative mRNA levels of EMT-related genes were normalized to GAPDH mRNA levels. Results are expressed as fold change against wild-type cells. Results shown represent mean $\pm$ SD, $n=3$.

of ECM (51). The association of MnSOD expression with the transition to a more mesenchymal functional phenotype is further supported by strong correlation between the drug resistance-induced mesenchymal phenotype and increased MnSOD expression in DOX-R MDA-MB-231 cells.

Collectively, results presented here underscore the critical role of MnSOD in regulating MET and EMT and thereby the process of metastasis and tumor progression. This is the first time that MnSOD is being implicated in EMT regulation in breast cancer, besides a recent report that manipulation of miR-212 altered levels of MnSOD and hence affected EMT traits in colorectal cancer (31). Manipulation of MnSOD protein levels leading to changes in epithelial and mesenchymal phenotypes in breast cancer cell lines also highlights the interchangeable and dynamic nature of the EMT program, whereby tumor cells could switch from an epithelial phenotype to a mesenchymal phenotype and vice versa, thus facilitating metastasis and tumor formation at secondary sites.

MnSOD promotes EMT by altering the intracellular redox environment

Previous studies have identified oxidative stress within the tumor microenvironment as a stimulus for the induction of EMT and development of an invasive phenotype during metastasis (6, 48). A direct cross talk between ROS and transforming growth factor beta (TGF- β) signaling was one of the earliest reported links between ROS and EMT; Rhyu *et al.* reported that TGF- β -dependent ROS induction was responsible for phosphorylating Smad2, MAPK, and ERK, upregulating Fibronectin, and repressing the expression of E-cadherin. H₂O₂ treatment reproduced the same EMT-related molecular effects, supporting the involvement of ROS. (38). In agreement with the previous study, H₂O₂ treatment was also reported to induce Snail-mediated hypermethylation of the E-cadherin promoter by recruitment of HDAC1 and DNA methyltransferase 1 (28). Meng *et al.* reported that high levels

of H₂O₂ activated the hypoxia-inducible factor 1 α , which enhanced the conferment of EMT traits on tumor cells (30).

Since MnSOD is a critical regulator of mitochondrial ROS, there is strong evidence to link MnSOD-induced changes in redox status to regulation of the EMT process. Indeed, our results in MnSOD-overexpressing MCF7 cells corroborate the involvement of H₂O₂ in promoting the EMT program. Conversely, suppression of MnSOD translates to lower H₂O₂ levels and accumulation of O₂^{•-}. O₂^{•-} then combines with NO to form the reactive nitrogen species, OONO⁻ (52), which like its precursor NO, could also be a potential inhibitor of EMT (2,5). Indeed, our data using FeTPPS, a scavenger of OONO⁻, provide strong evidence for a regulatory role of intracellular OONO⁻ on the EMT program upon suppression of MnSOD in breast cancer cells.

Concluding remarks

The ability of MnSOD to regulate breast cancer phenotype and functional biology is a function of its redox modulating activity *via* the dismutation of O₂^{•-} to H₂O₂. Consequently, any significant change in the expression levels of MnSOD alters the redox milieu *vis-a-vis* the O₂^{•-}:H₂O₂. In this regard, we have previously shown that not only does the intracellular O₂^{•-}:H₂O₂ dictate cell fate decisions (26), but also the signaling pathways engaged upon a tilt toward one or the other might involve different cellular processes with distinct physiological outcomes. Based on the observations reported in this communication, we contend that the varied expression of MnSOD in subtypes of breast cancers with different phenotypes and functional characteristics is a function of whether the redox signaling is predominantly O₂^{•-} driven or H₂O₂ mediated. The proliferative subtypes tend to be those in which the signaling is dictated by O₂^{•-} to support the MET program, while a switch to H₂O₂ is associated with the acquisition of invasive and migratory capabilities with hallmarks consistent with EMT (Fig. 7F). As such, MnSOD presents as a potential therapeutic target for designing strategic therapies against metastatic cancers as well as those that have become refractory to treatment.

Materials and Methods

Data preprocessing of affymetrix microarray gene expression

Invasive ductal breast cancer data were downloaded from The Cancer Genome Atlas (<http://cancergenome.nih.gov>). For this study, we included 536 breast tumor gene expression data available on Agilent custom gene expression microarray G4502A_07 platform. The data were imported to Partek[®] Genomics Suite 6.6 where the log ratio of gene expression value was quantile normalized for further analysis. Breast cancer cell line gene expression data were downloaded from GSE15026 and E-TABM-157 from Gene Expression Omnibus (<http://ncbi.nlm.nih.gov/gds>) and ArrayExpress (<http://ebi.ac.uk/arrayexpress/>), respectively. Robust Multichip Average normalization was performed on each dataset using R affy package version 1.42.3. The normalized data were compiled and subsequently standardized using ComBat (22) to alleviate batch effect.

FIG. 6. DOX-resistant MDA-MB-231 exhibits increased expression of EMT markers and migratory and invasive capacity. Expression of EMT markers was analyzed in both parental MDA-MB-231 (Parental) and its DOX-resistant derivative (DOX-R) cells. Western blot analysis is shown for (A) Vimentin, (B) N-Cadherin, (C) β -catenin, (D) Snail, and (E) Slug. MnSOD protein expression levels in parental and in DOX-R cells are also shown. Note that the expression of MnSOD in the parental cell line is relative to the DOX-R. β -Actin was used as an endogenous control. (F) Migration assay of parental MDA-MB-231 *versus* DOX-R cells. Cells were seeded in 60-mm dishes and grown to 90% confluence. A wound was made and images were taken at 0, 24, and 36 h to depict wound healing ability. (G) *Top*: Western blot to show protein levels of MnSOD. *Bottom*: The wound length between cells was measured at three separate regions. Results shown represent mean $\pm$ SD, $n = 3$. (H) Invasion assay of parental MDA-MB-231 *versus* DOX-R cells. Cells were seeded into the upper chamber of a transwell invasion chamber with serum-free media and grown for 24 h. The membranes of the chambers were then stained with crystal violet solution and mounted on glass slides. Representative light microscopy images ($\times 100$) are shown. (I) *Top*: Western blot analysis to show protein levels of MnSOD. *Bottom*: The number of cells that invaded through the transwell invasion chambers was counted in five randomly selected microscope fields. Results shown represent mean $\pm$ SD, $n = 3$. DOX, doxorubicin.

TABLE 4. ACTIVATION OF THE MESENCHYMAL–EPITHELIAL TRANSITION PHENOTYPE FOLLOWING A DECREASE IN MANGANESE SUPEROXIDE DISMUTASE EXPRESSION IS DEPENDENT ON PRODUCTION OF PEROXYNITRITE

	Fold change from ctsi/medium $\pm$ SD (n=3)		
	ctsi/ FeTPPS	siMnSOD/ medium	siMnSOD/ FeTPPS
MDA-MB-231			
N-Cadherin	1.53 $\pm$ 0.07	0.60 $\pm$ 0.06	1.35 $\pm$ 0.03
Vimentin	1.63 $\pm$ 0.07	0.65 $\pm$ 0.06	1.29 $\pm$ 0.01
Snail	3.02 $\pm$ 0.02	0.47 $\pm$ 0.05	2.20 $\pm$ 0.10

MDA-MB-231 cells were transfected with control scrambled RNA (ctsi) or 100 nM siMnSOD. Post-transfection, cells were incubated with and without 50 μ M FeTPPS for 48 h. Real-time PCR of EMT-associated genes in MDA-MB-231 cells was conducted. Relative mRNA levels of N-Cadherin, Vimentin, and Snail were normalized to GAPDH mRNA levels. Results are expressed as fold change against ctsi-transfected cells kept in control medium (ctsi/medium). Results shown represent mean $\pm$ SD, n = 3.

FeTPPS, 5,10,15,20-Tetrakis(4-sulfonatophenyl) porphyrinato iron (III) chloride.

Estimation of EMT score

An EMT scoring method was developed previously (43) to quantitate EMT from -1.0 to $+1.0$, where the extremes correspond to the most epithelial and most mesenchymal states, respectively. Briefly, a breast cancer EMT signature was derived comparing most epithelial and most mesenchymal breast cancer cell lines using significance analysis of microarray and receiver operating characteristic curve. With the signature, Single Sample Gene Set Enrichment Analysis [ssGSEA; (46)] was used to obtain the enrichment score for the EMT signature. EMT score is referred to as the normalized subtraction of the mesenchymal enrichment score from the epithelial enrichment score.

Identification of breast cancer subtypes

Breast cancer subtype was inferred based on gene expression pattern using ssGSEA and a subtype signature (37), as previously described (26). Briefly, using the breast cancer subtype signature, ssGSEA was used to compute the subtype enrichment scores of each sample. The sample is assigned the subtype with the highest enrichment score.

Cell lines and culture conditions

MDA-MB-231, BT-549, MCF7, T47D, and BT474 breast cancer cells (American Type Culture Collection) were used. Cell lines were routinely maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 0.05 mg/ml gentamicin in a 37°C incubator with 5% CO₂. The generation of DOX-resistant MDA-MB-231 cells was carried out as described previously (26).

Treatment of cells with ROS scavengers

NAC and FeTPPS were purchased from Sigma. Stock solutions of 1 M NAC were prepared in dimethyl sulfoxide (DMSO). Stock solutions of 50 mM FeTPPS were prepared in DMSO. Various concentrations of drugs were then prepared

by diluting the stock solution with complete medium to attain the desired final concentrations of 10 mM for NAC and 50 μ M FeTPPS.

Western blot analysis

Western blot was performed as described previously (26). Details are described in the Supplementary Materials and Methods section.

RNA isolation, reverse transcription, and real-time polymerase chain reaction

RNA isolation, reverse transcription, and real-time polymerase chain reaction (RT-PCR) were performed as described previously (26, 42). TaqMan primers for human 18S and MnSOD were purchased from ABI (Applied Biosystems). Fold change is calculated by $2^{(-\Delta\Delta C_t)}$, where $\Delta\Delta C_t = \Delta C_t$ (treatment) $- \Delta C_t$ (control) and $\Delta C_t = C_t$ (target gene) $- C_t$ (endogenous gene).

RT-PCR using SYBR green primers

Forward and reverse primers were used for RT-PCR using SYBR Green PCR Master Mix as previously described (42). Sequences are found in the Supplementary Materials and Methods section. SYBR Green RT-PCR was performed using 2X SYBR Green PCR Master Mix using ABI PRISM 7500 (Applied Biosystems). Fluorescence was measured with the Sequence Detection Systems 2.0 software.

DNA and siMnSOD transfection

For silencing of MnSOD, 100 nM of MnSOD siRNA 5'-AAAUUGCUGCUUGUCCAAAUUC-3' (16) or scrambled control siRNA 5'-AGCUUCAUAAGGCGCAUGCTT-3' (luciferase gene sequence inverted) was transiently transfected using LipofectAMINE RNAiMAX (Invitrogen) following the manufacturer's protocols.

Three-dimensional invasion assay

BD Bio-Coat Matrigel invasion assay system (BD Biosciences) was used to determine invasive capacity *in vitro*, as previously described (42). Briefly, cells were trypsinized 48 h post-transfection, 1.5×10^5 cells in serum-free culture medium, and plated into the Matrigel transwell chambers containing polycarbonate membranes with 8 μ m pores. Regular medium containing 10% FBS was added to the bottom chamber to act as chemoattractant. After incubation for 48 h, the media in the transwell chamber were removed, and upper surface of the chambers was wiped off with a cotton swab. The invading cells were fixed and stained with crystal violet solution. The invading cell numbers were counted in five randomly selected microscope fields ($\times 200$) and their averages were converted to percentage with the control setup taken to be at 100%.

Cell scattering assay

MDA-MB-231, BT549, and MCF7 cells were subjected to MnSOD knockdown or overexpression. After 48 h, cells were trypsinized and counted. One thousand cells were then reseeded into six-well plates and allowed to grow for 2 weeks. Colonies formed from at least 30 cells were then classified

FIG. 7. Redox control of the switch between an EMT and an MET-like phenotype in breast carcinoma. MDA-MB-231 cells were transfected with control scrambled RNA (ctsi) or 100 nM siMnSOD. Post-transfection, cells were incubated with and without 50 μ M FeTPPS for 48 h. (A) Invasion assay of MDA-MB-231 $\pm$ 50 μ M FeTPPS for 48 h. Cells were then trypsinized and seeded into the upper chamber of a transwell invasion chamber with serum-free media and grown for 24 h. The membranes of the chambers were then stained with crystal violet solution and mounted on glass slides. The number of cells that invaded through the transwell invasion chambers was counted in five randomly selected microscope fields. (B) Migration assay of MDA-MB-231 transfected with control scrambled RNA (ctsi) or 100 nM siMnSOD and incubated with and without 50 μ M FeTPPS for 48 h. Cells were then trypsinized and seeded in 60-mm dishes and grown to 90% confluence. A wound was made and images were taken at 0, 24, and 48 h to depict wound healing ability. (C) *Top*: Western blot to show knockdown of MnSOD. *Bottom*: The wound length between cells was measured at three separate regions. (D) The EMT-promoting activity of MnSOD is linked to its ability to alter the intracellular redox milieu, in particular an imbalance in the $O_2^{\cdot-}$: H_2O_2 ratio in favor of H_2O_2 , while the MET-promoting effect is associated with an imbalance of the $O_2^{\cdot-}$: H_2O_2 ratio in favor of $O_2^{\cdot-}$. FeTPPS, 5,10,15,20-Tetrakis(4-sulfonatophenyl) porphyrinato iron (III) chloride; H_2O_2 , hydrogen peroxide; MET, mesenchymal-epithelial transition; $O_2^{\cdot-}$, superoxide.

TABLE 5. ACTIVATION OF THE EPITHELIAL–MESENCHYMAL TRANSITION PHENOTYPE FOLLOWING AN INCREASE IN MANGANESE SUPEROXIDE DISMUTASE EXPRESSION IS DEPENDENT ON AN INCREASE IN REACTIVE OXYGEN SPECIES, MOST LIKELY HYDROGEN PEROXIDE

MCF7	Fold change from pcDNA3/medium $\pm$ SD (n = 3)		
	pcDNA3/NAC	pcDNA3-MnSOD/medium	pcDNA3-MnSOD/NAC
E-cadherin	0.75 $\pm$ 0.10	0.35 $\pm$ 0.13	1.29 $\pm$ 0.16
N-Cadherin	1.05 $\pm$ 0.50	3.67 $\pm$ 0.02	1.27 $\pm$ 0.13
Fibronectin	0.83 $\pm$ 0.08	1.74 $\pm$ 0.16	1.27 $\pm$ 0.07

MCF7 cells were transfected with pcDNA3 vector or pcDNA3-MnSOD plasmid. Post-transfection, cells were incubated with 5 mM NAC for 48 h. Real-time PCR of EMT-associated genes was conducted. Relative mRNA levels of E-Cadherin, N-Cadherin, and Fibronectin were normalized to GAPDH mRNA levels. Results are expressed as fold change against pcDNA3-transfected cells kept in control medium (pcDNA3/medium). Results shown represent mean $\pm$ SD, n = 3.

NAC, N-acetyl cysteine.

into three groups (tight, loose, and scattered) based on intercellular distance between cells in a colony. Numbers of colonies were then counted and expressed as percentages of the total number of colonies formed.

Wound healing assay

MDA-MB-231 and BT549 cells were treated according to the experimental design and a wound healing assay was carried out as described (29, 42). Briefly, two parallel lines were drawn at the underside of the well before plating the cells. These lines serve as fiducial marks for the analysis of the wound areas. The cells were 90% confluent before marking the wound. The growth medium was replaced with calcium-free phosphate-buffered saline (PBS) to prevent calcium-mediated cell death at the edge of the wound. Two parallel scratch wounds were made perpendicular to the marker lines with a 200 μ l yellow tip. The medium was then changed to complete media. After incubation for 24–72 h, multiple images of the region juxtaposed to the intersection of the wound area and the marker lines (at the beginning and end of the experiment) were taken by bright-field microscopy. Using these images, three measurements of the gap area between the wound were taken at the start and end of the experiment and their averages were converted to percentages to depict percentage change with the control setup taken as 100%.

Immunofluorescence staining

For immunofluorescence assay, cells were seeded on coverslips in 24-well plates. Cells were fixed with 100% methanol for 30 min at room temperature. After fixation, cells were washed with 1 $\times$ PBS twice to remove excess methanol. Cells were permeabilized with 0.2% TritonX-100 (Sigma-Aldrich) for 10 min at room temperature. Cells were then incubated with primary antibody of the protein of interest overnight at 4°C. After washing with PBS, cells were incubated with Alexa Fluor 568 goat anti-mouse acid immunoglobulin G (Molecular Probes, Inc., Eugene) and with 4', 6-diamidino-2-phenylindole nucleic stain (Molecular Probes, Inc., Eugene) for 1 min. The cells were washed three times with 1 $\times$ PBS before the coverslip was mounted for confocal analysis using Olympus FluoView1000 (FV1000; Olympus) with identical acquisition parameters for the same image session.

Pictures are analyzed with Olympus FLUOVIEW Ver1.7a Viewer.

Statistical analysis

Statistical analysis was performed using paired Student's *t*-test. A *p*-value of <0.05 was considered significant. Statistical significance evaluation for microarray analysis was performed using the Mann–Whitney test and Spearman correlation test, which were computed using Matlab® R2012a (MathWorks). Dot plots were done using GraphPad Prism® version 5.0 (GraphPad Software).

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Author Contributions

S.Y.L., J.L.H. and V.P. contributed to the generation of data. P.E.L. contributed reagents to perform the experiments. T.Z.T. and J.P.T. contributed to the data presented in Figure 1. T.Z.T. generated Figure 1. S.P., M.V.C. and A.P.K. directed the research and wrote the manuscript. M.V.C. and S.P. contributed to the redox concept presented in this manuscript. M.V.C. generated Figure 7D. J.L.H. and V.P. performed additional experiments suggested by reviewers. C.T.Y. and B.C.G. supported S.Y.L. and J.L.H., and A.P.K. salaries. All authors have agreed to the authorship and approved the manuscript.

Author Disclosure Statement

No competing financial interests exist.

References

1. Aghdassi A, Sendler M, Guenther A, Mayerle J, Behn CO, Heidecke CD, Friess H, Buchler M, Evert M, Lerch MM, and Weiss FU. Recruitment of histone deacetylases HDAC1 and HDAC2 by the transcriptional repressor ZEB1 downregulates E-cadherin expression in pancreatic cancer. *Gut* 61: 439–448, 2012.
2. Baritaki S, Huerta-Yepez S, Sahakyan A, Karagiannides I, Bakirtzi K, Jazirehi A, and Bonavida B. Mechanisms of nitric oxide-mediated inhibition of EMT in cancer: inhibition of the metastasis-inducer Snail and induction of the metastasis-suppressor RKIP. *Cell Cycle* 9: 4931–4940, 2010.
3. Blanco MJ, Moreno-Bueno G, Sarrio D, Locascio A, Cano A, Palacios J, and Nieto MA. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* 21: 3241–3246, 2002.
4. Blick T, Widodo E, Hugo H, Waltham M, Lenburg ME, Neve RM, and Thompson EW. Epithelial mesenchymal transition traits in human breast cancer cell lines. *Clin Exp Metastasis* 25: 629–642, 2008.
5. Bonavida B and Baritaki S. Dual role of NO donors in the reversal of tumor cell resistance and EMT: downregulation of the NF-kappaB/Snail/YY1/RKIP circuitry. *Nitric Oxide* 24: 1–7, 2011.
6. Cannito S, Novo E, di Bonzo LV, Busletta C, Colombatto S, and Parola M. Epithelial-mesenchymal transition: from molecular mechanisms, redox regulation to implications in human health and disease. *Antioxid Redox Signal* 12: 1383–1430, 2010.
7. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, and Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2: 76–83, 2000.
8. Chen PM, Wu TC, Shieh SH, Wu YH, Li MC, Sheu GT, Cheng YW, Chen CY, and Lee H. MnSOD promotes tumor invasion via upregulation of FoxM1-MMP2 axis and related with poor survival and relapse in lung adenocarcinomas. *Mol Cancer Res* 11: 261–271, 2013.
9. Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, and Wang LH. Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. *Cancer Res* 67: 1979–1987, 2007.
10. Cruz P, Epanan MJ, Ramirez ME, Torres CG, Valladares LE, and Sierralta WD. 27-Hydroxycholesterol and the expression of three estrogen-sensitive proteins in MCF7 cells. *Oncol Rep* 28: 992–998, 2012.
11. Dhar SK and St. Clair DK. Manganese superoxide dismutase regulation and cancer. *Free Radic Biol Med* 52: 2209–2222, 2012.
12. Dhar SK, Tangpong J, Chaiswing L, Oberley TD, and St. Clair DK. Manganese superoxide dismutase is a p53-regulated gene that switches cancers between early and advanced stages. *Cancer Res* 71: 6684–6695, 2011.
13. Echiburu-Chau C, Roy D, and Calaf GM. Metastatic suppressor CD44 is related with oxidative stress in breast cancer cell lines. *Int J Oncol* 39: 1481–1489, 2011.
14. Ennen M, Minig V, Grandemange S, Touche N, Merlin JL, Besancenot V, Brunner E, Domenjoud L, and Becuwe P. Regulation of the high basal expression of the manganese superoxide dismutase gene in aggressive breast cancer cells. *Free Radic Biol Med* 50: 1771–1779, 2011.
15. Fazilaty H, Gardaneh M, Bahrami T, Salmaninejad A, and Behnam B. Crosstalk between breast cancer stem cells and metastatic niche: emerging molecular metastasis pathway? *Tumour Biol* 34: 2019–2030, 2013.
16. Funasaka T, Hu H, Hogan V, and Raz A. Down-regulation of phosphoglucose isomerase/autocrine motility factor expression sensitizes human fibrosarcoma cells to oxidative stress leading to cellular senescence. *J Biol Chem* 282: 36362–36369, 2007.
17. Gao D, Vahdat LT, Wong S, Chang JC, and Mittal V. Microenvironmental regulation of epithelial-mesenchymal transitions in cancer. *Cancer Res* 72: 4883–4889, 2012.
18. Giannoni E, Parri M, and Chiarugi P. EMT and oxidative stress: a bidirectional interplay affecting tumor malignancy. *Antioxid Redox Signal* 16: 1248–1263, 2012.
19. Halliwell B and Gutteridge J. *Free Radicals in Biology and Medicine*. New York: Oxford University Press, 1999.
20. Holley AK, Kinningham KK, Spitz DR, Edwards DP, Jenkins JT, and Moore MR. Progestin stimulation of manganese superoxide dismutase and invasive properties in T47D human breast cancer cells. *J Steroid Biochem Mol Biol* 117: 23–30, 2009.
21. Jiang J, Tang YL, and Liang XH. EMT: a new vision of hypoxia promoting cancer progression. *Cancer Biol Ther* 11: 714–723, 2011.
22. Johnson WE, Li C, and Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 8: 118–127, 2007.
23. Jones PL, Ping D, and Boss JM. Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB. *Mol Cell Biol* 17: 6970–6981, 1997.
24. Kamarajugadda S, Cai Q, Chen H, Nayak S, Zhu J, He M, Jin Y, Zhang Y, Ai L, Martin SS, Tan M, and Lu J. Manganese superoxide dismutase promotes anoikis resistance and tumor metastasis. *Cell Death Dis* 4: e504, 2013.
25. Kattan Z, Minig V, Leroy P, Dauca M, and Becuwe P. Role of manganese superoxide dismutase on growth and invasive properties of human estrogen-independent breast cancer cells. *Breast Cancer Res Treat* 108: 203–215, 2008.
26. Kumar AP, Loo SY, Shin SW, Tan TZ, Eng CB, Singh R, Putti TC, Ong CW, Salto-Tellez M, Goh BC, Park JJ, Thierry JP, Pervaiz S, and Clement MV. Manganese superoxide dismutase is a promising target for enhancing chemosensitivity of basal-like breast carcinoma. *Antioxid Redox Signal* 20: 2326–2346, 2014.
27. Li F, Wang H, Huang C, Lin J, Zhu G, Hu R, and Feng H. Hydrogen peroxide contributes to the manganese superoxide dismutase promotion of migration and invasion in glioma cells. *Free Radic Res* 45: 1154–1161, 2011.
28. Lim SO, Gu JM, Kim MS, Kim HS, Park YN, Park CK, Cho JW, Park YM, and Jung G. Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. *Gastroenterology* 135: 2128–2140, 2140e1–e8, 2008.
29. Lin KL, Chien CM, Hsieh CY, Tsai PC, Chang LS, and Lin SR. Antimetastatic potential of cardiotoxin III involves inactivation of PI3K/Akt and p38 MAPK signaling pathways in human breast cancer MDA-MB-231 cells. *Life Sci* 90: 54–65, 2012.
30. Meng FJ, Hou ZW, Li Y, Yang Y, and Yu B. The protective effect of picoside II against hypoxia/reoxygenation

- injury in neonatal rat cardiomyocytes. *Pharm Biol* 50: 1226–1232, 2012.
31. Meng X, Wu J, Pan C, Wang H, Ying X, Zhou Y, Yu H, Zuo Y, Pan Z, Liu RY, and Huang W. Genetic and epigenetic down-regulation of microRNA-212 promotes colorectal tumor metastasis via dysregulation of MnSOD. *Gastroenterology* 145: 426–436e1–e6, 2013.
 32. Molina-Ortiz P, Villarejo A, MacPherson M, Santos V, Montes A, Souchelnytskyi S, Portillo F, and Cano A. Characterization of the SNAG and SLUG domains of Snail2 in the repression of E-cadherin and EMT induction: modulation by serine 4 phosphorylation. *PLoS One* 7: e36132, 2012.
 33. Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, Notorfrancesco KL, Cardiff RD, and Chodosh LA. The transcriptional repressor Snail promotes mammary tumor recurrence. *Cancer Cell* 8: 197–209, 2005.
 34. Mukhopadhyay S, Das SK, and Mukherjee S. Expression of Mn-superoxide dismutase gene in nontumorigenic and tumorigenic human mammary epithelial cells. *J Biomed Biotechnol* 4: 195–202, 2004.
 35. Nam EH, Lee Y, Park YK, Lee JW, and Kim S. ZEB2 upregulates integrin alpha5 expression through cooperation with Sp1 to induce invasion during epithelial-mesenchymal transition of human cancer cells. *Carcinogenesis* 33: 563–571, 2012.
 36. Pan X, Wang X, Lei W, Min L, Yang Y, and Song J. Nitric oxide suppresses transforming growth factor-beta1-induced epithelial-to-mesenchymal transition and apoptosis in mouse hepatocytes. *Hepatology* 50: 1577–1587, 2009.
 37. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X, and Perou CM. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res* 12: R68, 2010.
 38. Rhyu DY, Yang Y, Ha H, Lee GT, Song JS, Uh ST, and Lee HB. Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol* 16: 667–675, 2005.
 39. Roxanis I. Occurrence and significance of epithelial-mesenchymal transition in breast cancer. *J Clin Pathol* 66: 517–521, 2013.
 40. Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, and Palacios J. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res* 68: 989–997, 2008.
 41. Shah P, Gau Y, and Sabnis G. Histone deacetylase inhibitor entinostat reverses epithelial to mesenchymal transition of breast cancer cells by reversing the repression of E-cadherin. *Breast Cancer Res Treat* 143: 99–111, 2014.
 42. Shin EM, Hay HS, Lee MH, Goh JN, Tan TZ, Sen YP, Lim SW, Yousef EM, Ong HT, Thike AA, Kong X, Wu Z, Mendoz E, Sun W, Salto-Tellez M, Lim CT, Lobie PE, Lim YP, Yap CT, Zeng Q, Sethi G, Lee MB, Tan P, Goh BC, Miller LD, Thiery JP, Zhu T, Gaboury L, Tan PH, Hui KM, Yip GW, Miyamoto S, Kumar AP, and Tergaonkar V. DEAD-box helicase DP103 defines metastatic potential of human breast cancers. *J Clin Invest* 124: 3807–3824, 2014.
 43. Tan TZ, Miow QH, Miki Y, Noda T, Mori S, Huang RY, and Thiery JP. Epithelial-mesenchymal transition spectrum quantification and its efficacy in deciphering survival and drug responses of cancer patients. *EMBO Mol Med* 6: 1279–1293, 2014.
 44. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2: 442–454, 2002.
 45. Tsai YP and Wu KJ. Hypoxia-regulated target genes implicated in tumor metastasis. *J Biomed Sci* 19: 102, 2012.
 46. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, Getz G, Perou CM, and Hayes DN. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17: 98–110, 2010.
 47. Wang Y and Shang Y. Epigenetic control of epithelial-to-mesenchymal transition and cancer metastasis. *Exp Cell Res* 319: 160–169, 2013.
 48. Wang Z, Li Y, and Sarkar FH. Signaling mechanism(s) of reactive oxygen species in epithelial-mesenchymal transition reminiscent of cancer stem cells in tumor progression. *Curr Stem Cell Res Ther* 5: 74–80, 2010.
 49. Xu W, Lacerda L, Debeb BG, Atkinson RL, Solley TN, Li L, Orton D, McMurray JS, Hang BI, Lee E, Klopp AH, Ueno NT, Reuben JM, Krishnamurthy S, and Woodward WA. The antihelminthic drug pyriminium pamoate targets aggressive breast cancer. *PLoS One* 8: e71508, 2013.
 50. Xu Y, Kiningham KK, Devalaraja MN, Yeh CC, Majima H, Kasarskis EJ, and St. Clair DK. An intronic NF-kappaB element is essential for induction of the human manganese superoxide dismutase gene by tumor necrosis factor-alpha and interleukin-1beta. *DNA Cell Biol* 18: 709–722, 1999.
 51. Zhang HJ, Zhao W, Venkataraman S, Robbins ME, Buettner GR, Kregel KC, and Oberley LW. Activation of matrix metalloproteinase-2 by overexpression of manganese superoxide dismutase in human breast cancer MCF-7 cells involves reactive oxygen species. *J Biol Chem* 277: 20919–20926, 2002.
 52. Zielonka J, Sikora A, Joseph J, and Kalyanaraman B. Peroxynitrite is the major species formed from different flux ratios of co-generated nitric oxide and superoxide: direct reaction with boronate-based fluorescent probe. *J Biol Chem* 285: 14210–14216, 2010.
 53. Zucchini-Pascal N, Peyre L, and Rahmani R. Crosstalk between beta-catenin and snail in the induction of epithelial to mesenchymal transition in hepatocarcinoma: role of the ERK1/2 pathway. *Int J Mol Sci* 14: 20768–20792, 2013.

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Abbreviations Used

BSA = bovine serum albumin
DAPI = 4',6-diamidino-2-phenylindole
DMSO = dimethyl sulfoxide
DOX = doxorubicin
ECM = extracellular matrix
EMT = epithelial-mesenchymal transition
ERK1/2 = extracellular signal-regulated kinases 1/2
FBS = fetal bovine serum
FeTPPS = 5,10,15,20-Tetrakis(4-sulfonatophenyl)
porphyrinato iron (III) chloride

FoxM1 = Forkhead box M1
FoxO = Forkhead box O1
H₂O₂ = hydrogen peroxide
HDAC1 = histone deacetylase 1
HDAC2 = histone deacetylase 2
IgG = immunoglobulin G
MAPK = mitogen-activated protein kinase
MET = mesenchymal-epithelial transition
MMP = matrix metalloproteinase
MnSOD = manganese superoxide dismutase
NAC = N-acetyl cysteine
NO = nitric oxide
O₂^{•-} = superoxide
OONO = peroxynitrite
PBS = phosphate-buffered saline
ROS = reactive oxygen species
RT-PCR = real-time polymerase chain reaction
SD = standard deviation
Sp1 = specificity protein 1
TCGA = The Cancer Genome Atlas
TF = transcription factor
TGF- β = transforming growth factor beta
TNBC = triple negative breast cancer
Wnt = Wingless-type MMTV integration site family
Zeb1 = zinc finger E-Box binding homeobox 1
Zeb2 = zinc finger E-Box binding homeobox 2