Published in final edited form as:

Mol Cancer Res. 2012 January ; 10(1): 40–51. doi:10.1158/1541-7786.MCR-11-0501.

Genetic and epigenetic inactivation of extracellular superoxide dismutase promotes an invasive phenotype in human lung cancer by disrupting ECM homeostasis

Melissa L.T. Teoh^{1,*}, Matthew P. Fitzgerald^{1,*}, Taylor J. Jensen^{2,3}, Bernard W. Futscher², and Frederick E. Domann^{1,#}

¹Free Radical and Radiation Biology Program, Department of Radiation Oncology, Carver College of Medicine and The Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, 52242

²Department of Pharmacology and Toxicology, College of Pharmacy, and The Arizona Cancer Center, University of Arizona, Tucson, AZ 85724

Abstract

Extracellular superoxide dismutase (*EcSOD*) is an important superoxide scavenger in the lung where its loss, sequence variation, or abnormal expression contributes to lung diseases; however, the role of *EcSOD* in lung cancer has yet to be studied. We hypothesized that *EcSOD* loss could affect malignant progression in lung, and could be either genetic or epigenetic in nature. To test this we analyzed *EcSOD* expression, gene copy number, promoter methylation and chromatin accessibility in normal lung and carcinoma cells. We found that normal airway epithelial cells expressed abundant *EcSOD* and had an unmethylated promoter, whereas *EcSOD*-negative lung cancer cells displayed aberrant promoter hypermethylation and decreased chromatin accessibility. 5-aza-dC induced *EcSOD* suggesting that cytosine methylation was causal, in part, to silencing. In 48/50 lung tumors *EcSOD* mRNA was significantly lower as early as stage I, and the *EcSOD* promoter was hypermethylated in 8/10 (80%) adenocarcinomas compared to 0/5 normal lung samples. In addition, 20% of the tumors showed LOH of *EcSOD*. Re-expression of *EcSOD* attenuated the malignant phenotype of lung carcinoma cells by significantly decreasing invasion and survival. Finally, *EcSOD* decreased heparanase and syndecan-1 mRNAs in part by reducing NF- κ B. By contrast, MnSOD and CuZnSOD showed no significant changes in lung tumors and had no effect on heparanase expression. Taken together, the loss of *EcSOD* expression is unique among the superoxide dismutases in lung cancer and is the result of *EcSOD* promoter methylation and LOH, suggesting that its early loss may contribute to ECM remodeling and malignant progression.

Keywords

SOD3; *EcSOD*; Heparanase; Syndecan-1; NF- κ B; DNA methylation

[#]Corresponding author: Frederick E. Domann, Free Radical and Radiation Biology Program, B180 Medical Laboratories, University of Iowa, Iowa City, IA 52242, USA. Frederick-Domann@uiowa.edu.

³Current address: Sequenom Center for Molecular Medicine, 3595 John Hopkins Ct., San Diego, CA, 92121

*These authors are co-first authors and contributed equally to this work

Introduction

Extracellular Superoxide Dismutase, *EcSOD* (also known as SOD3) is the predominant scavenger of superoxide in the extracellular space and functions in part to protect the bioavailability of nitric oxide (NO) (1). *EcSOD* displays a cell-type specific pattern of expression and is most abundantly expressed in lung, pancreas, and vasculature tissue (2).

In the human lung, *EcSOD* is highly expressed in airway epithelial and vascular endothelial cells and has been shown to comprise a large fraction of all lung SOD activity (3). The lungs are unique in their exposure to environmental oxidants and for this reason the majority of investigations have focused on *EcSOD*'s role in pulmonary diseases such as COPD and its protective role against infection, inflammation and environmental pollutants (4-5). In support of this role for *EcSOD* in oxidative-mediated lung injuries, *EcSOD* knockout mice have been shown to have enhanced lung damage and reduced survival when exposed to hyperoxia and bleomycin (6-7). Conversely, *EcSOD* overexpressing transgenic mice are more resistant to these oxidative insults compared to wild type mice (8).

EcSOD contains a heparin binding domain which enables it to bind to heparan sulfate (HS), hyaluronan, and Type I collagen (9). This unique property of *EcSOD* to bind to glycosaminoglycan constituents of the extracellular matrix has been studied in pulmonary mouse models and shown to protect against oxidative cleavage of HS, hyaluronan and collagen (10-12). Recently we reported that *EcSOD* protected HS cleavage in breast cancer cells (13). In humans, *EcSOD* has been shown to be significantly reduced in fibrotic areas of Idiopathic Pulmonary Fibrosis (IPF) and polymorphisms have been associated with decreased lung function, development and risk for COPD (14-15). Taken together these studies strongly implicate an important role of *EcSOD* in lung pathophysiology, however, to date few studies have examined *EcSOD*'s role in lung carcinogenesis. A recent study in a limited number of samples (five tumors) discovered that *EcSOD* protein was significantly decreased or lost in both clinical squamous cell and adenocarcinoma lung tumors (16). These data were striking because of the reliance of the lung on *EcSOD* to scavenge extracellular superoxide. This is highlighted by the fact that the highest level of oxygen tension in the body is at the alveolar surface which is a central location for *EcSOD* in the lung. Recently Zelko, *et al.* reported that while the lung cancer cell line A549 has abundant SP1/SP3 transcription factors, known to regulate *EcSOD* gene expression, these factors could not bind to the *EcSOD* promoter nor stimulate its expression (17).

In light of these recent findings the aim of this study was to determine the frequency of loss of *EcSOD* expression in lung cancer cells and the mechanism behind this loss of *EcSOD*. We hypothesized that cytosine methylation of the 5'-end of the *EcSOD* gene, particularly near the SP1/SP3 site, together with a more compact chromatin structure could lead to the epigenetic silencing of *EcSOD* in lung cancer and that re-acquisition of *EcSOD* might attenuate its malignant phenotype.

In addition to DNA promoter methylation analysis, the status of allelic loss manifested as LOH or genetic alterations is important in determining gene inactivation. The *EcSOD* gene has been mapped to chromosome 4p15.3-4p15.1(18). Previous reports using microsatellite markers at 4p15.3-4p15.1 show LOH of 63% in mesothelioma, 60% in SCLC, 63% in breast carcinomas, and 25% in NSCLC (19-21). In this study we examined whether LOH of *EcSOD* may also be a contributing factor to its downregulation in lung adenocarcinomas.

In this study we report that reduced expression of *EcSOD* in lung cancer cells and tissues is associated with aberrant CpG methylation of the *EcSOD* promoter and to a lesser extent to LOH. *EcSOD* mRNA expression was significantly lower than normal lung in 48/50 lung tumor samples and was shown to be significantly reduced as early as stage I with a further

reduction by stage IV. Furthermore, re-expression of EcSOD in A549 and H1650 lung carcinoma cells resulted in suppression of their *in vitro* clonogenic survival and invasion. We provide further evidence that these inhibitory effects of EcSOD are partly due to a decrease in expression of heparanase and syndecan-1 in part through suppression of NF- κ B activity. We also examined both MnSOD and CuZnSOD and found no significant changes in expression in lung tumors and discovered that forced overexpression of these superoxide dismutases had no effect on heparanase expression. This further highlighted the importance that loss of EcSOD may be playing in lung cancer redox biology due to its unique extracellular location and functions. To our knowledge, this study is the first to examine the role played by EcSOD in lung carcinogenesis.

Materials and Methods

Cell Culture and Clinical Tissue

Human mammary epithelial (HMEC) cells were obtained and maintained according to Cell Applications, Inc. Human airway epithelial cells (HAE) were obtained from The University of Iowa Center for Gene Therapy of Cystic Fibrosis. HAE cells are primary cultures of human bronchial epithelial cells grown on transwell membranes at an air:liquid interface; they are fully differentiated with tight junctions and microvilli (22). All human lung cell lines, A549, lung fibroblasts MRC-5, NCI-H1975 and NCI-H1650 were obtained from ATCC and maintained according to ATCC. The cell lines NCI-H1650 and NCI-H1975 were a generous gift from Dr. Paul Rothman (University of Iowa). Ten adenocarcinoma tumors of the lung, with four matched tumor:normal pairs were obtained from The University of Iowa Tissue Procurement Core Facility through an IRB approved protocol. In addition, samples of normal human lung total RNA and genomic DNA were obtained from Biochain Institute Inc. (catalogue numbers: R1234157 and D1234152, respectively).

Real-time reverse transcription-PCR analysis

EcSOD and heparanase expression were measured using TaqMan primer/ probe sets and syndecan-1 expression was measured using Power Sybr Green PCR master mix (Applied Biosystems, Inc.) Real-time RT-PCR reactions were performed with primers listed in Supplemental Table 1, and differences in expression were determined as previously described (23).

Chromatin Accessibility

Chromatin accessibility experiments were conducted as previously described (23). Primers used are shown in Supplemental Table 1.

Sodium Bisulfite Sequencing

Sodium bisulfite sequencing for *EcSOD* was performed as previously described (23). The 18 CpG sites of the *EcSOD* promoter examined herein spanned 659 bp from -544 bp upstream from the transcriptional start site downstream to +115 bp. This correlates with nucleotide positions 24796689-24797348 of the Feb. 2009 assembly of the UCSC genome browser. Primers used are listed in Supplemental Table 1.

COBRA and COBRA + Real-Time PCR

Combined bisulfite restriction analyses employed the same primers as sodium bisulfite sequencing experiment to amplify *EcSOD* products. The amplified products were then restricted with the enzymes TaqI, SnaBI, and BstUI and run on 2% agarose gels. Primers were also designed surrounding the TaqI restriction site to determine methylation changes in tumor versus normal. Methylation differences were calculated using the following formula:

COBRA Methylation Index = $2^{((Ct \text{ TaqI treated}) - (Ct \text{ Uncut}))}$. Primers used are shown in Supplemental Table 1.

DNA Methylation by Sequenom MassArray Analysis

DNA methylation analysis was performed using Sequenom MassARRAY according to manufacturer's protocol. Primers used are shown in Supplemental Table 1.

TaqMan Copy Number Assay

Copy number variation was determined using Taqman Copy Number Assay specific to *EcSOD*. A RNase P reference assay was used as an in-well copy number control. Copy number assays were performed according to manufacturer's instructions (Applied Biosystems, Inc.). Copy numbers were determined using Applied Biosystems V1 CopyCaller software to compute confidence values and absolute z-scores to assess the reliability of calls.

Adenovirus EcSOD gene transduction

Overexpression of EcSOD was achieved by adenovirus infection (AdEcSOD), as previously described (24) with a multiplicity of infection (MOI) of 40 for 48h.

Clonogenic assays

After 48h of adenoviral vector infection, A549 cells were seeded for clonogenic assays as previously described (13).

Invasion assay

The *in vitro* invasive properties of the lung cancer cells A549 and H1650 were determined after infection with 40 MOI of AdEcSOD, AdEmpty or no treatment using the BD Bio-Coat Matrigel invasion assay system (BD Biosciences) as previously described (13).

Western blot analysis

EcSOD western blot was performed as previously described (24). The EcSOD antibody was a generous gift from Dr. James Crapo (National Jewish Medical and Research Center, Denver, CO).

Antioxidant enzyme activity gels

EcSOD activity gel was performed as previously described (13).

Lung Cancer qPCR Array

EcSOD mRNA expression in lung tumor samples ranging from Stage I to Stage IV was measured in a 96 well plate using the TissueScan RT Lung Cancer qPCR Array III, plate HLRT103, lot number 0809, from Origene, Inc. Real-Time PCR was conducted on an ABI 7000 according to manufacturer's instruction.

Statistical Analysis

Statistical analyses were performed using MYSTAT 12, for some experiments a one-way ANOVA followed by post-hoc Tukey's test was used for statistical difference between means. Statistical analyses were also assessed using a two-tailed Student's *t*-test.

Results

EcSOD gene expression is silent in human lung adenocarcinoma cell lines, but can be re-activated by 5-Aza-dC treatment

Compared to normal human airway epithelial (HAE) cells, A549 and H1650 human lung adenocarcinoma cells lines showed no detectable levels of EcSOD protein expression. The H1975 cell line retained EcSOD expression, thus loss of detectable expression was present in 2/3 lung adenocarcinoma cells lines examined (**Fig. 1A**). HMEC cells that do not normally express EcSOD in cell culture were used as a negative control. RT-PCR analysis (**Fig. 1B**) showed robust expression of EcSOD mRNA in normal human lung tissue (NL), MRC-5 lung fibroblasts, HAE, and H1975 cells, while in contrast A549, H1650, and HMEC cells had undetectable levels of EcSOD mRNA expression. These results indicate that the down-regulation of EcSOD expression is occurring at the level of transcription. To examine whether the *EcSOD* gene promoter in non-expressing cells was maintained in a more condensed chromatin structure we measured nuclease accessibility in the Sp1/Sp3 region of the *EcSOD* promoter in the EcSOD-positive H1975 and EcSOD-negative A549 and H1650 cells. The accessibility indices (AI) for A549 and H1650 cells were determined to be 5-fold and 3-fold less accessible, respectively, to nuclease digestion when compared to H1975 cells (**Fig. 1C**). These findings indicate that the EcSOD-negative cells (A549 and H1650) have a more condensed chromatin structure surrounding the Sp1/Sp3 region in the *EcSOD* promoter. To elucidate a possible mechanism for loss of EcSOD mRNA expression we treated the EcSOD-negative H1650 cells with a DNA methyltransferase inhibitor, 5-Aza-dC. As shown in **Fig. 1D**, we found a marked induction of EcSOD mRNA expression in 5-Aza-dC treated cells suggesting that aberrant cytosine methylation of the *EcSOD* promoter could be contributing to the loss of EcSOD expression in lung adenocarcinoma cells. Together, these results led us to examine directly whether cytosine methylation surrounding the Sp1/Sp3 in the *EcSOD* promoter is associated with this more closed chromatin state and reduced EcSOD mRNA expression

The *EcSOD* promoter is aberrantly methylated in lung adenocarcinoma cells that have lost EcSOD expression

To determine if cytosine methylation of *EcSOD* promoter region is associated with EcSOD transcriptional silencing, we performed sodium bisulfite sequencing to query the 18 CpG sites at the 5' end of the gene. The region of interest was surrounding the Sp1/Sp3 site from -550 to +100 bp from the putative transcription start site (**Fig. 2A**). Our results, shown in **Fig. 2B**, indicated that the EcSOD-positive cell lines HAE, MRC-5, and H1975 displayed low levels of cytosine methylation across this regulatory region of the *EcSOD* promoter with overall EcSOD promoter methylation percentages of 8%, 13%, and 12% respectively. In contrast, the EcSOD-negative A549, H1650, and HMEC cells displayed significantly greater percentages of 86%, 86% and 79%, respectively. These data confirmed preliminary results by COBRA we obtained in MRC-5 cells versus A549 cells (**Supplemental Fig. 2**). These data reveal a distinct inverse correlation between EcSOD expression and the cytosine methylation status of this regulatory region in the *EcSOD* promoter.

EcSOD repression is linked to aberrant *EcSOD* promoter methylation in clinical lung adenocarcinoma tumors

We next sought to determine whether the *in vitro* findings correlating EcSOD transcriptional silencing with promoter methylation could be extended *in vivo*. As shown in **Fig. 3A**, all ten (100%) of the human lung adenocarcinoma tumors initially examined showed decreased mRNA expression of EcSOD when compared to the average range of expression in 5 normal lung samples (*dotted lines*). In **Fig. 3B** the box-plot shows the significant difference ($P < .001$) in the mean expression levels of EcSOD mRNA between the 10 tumors versus the

5 normal lung samples. Four of these normal tissues were from unaffected adjacent normal lung tissue and therefore can be considered as normal matched pairs to the lung tumors (N7:T7, etc.). These matched normal:tumor pairs were analyzed for EcSOD mRNA expression and in each case the level of EcSOD was significantly lower in the patient's tumor sample compared to the adjacent normal tissue (**Fig. 3C**). We additionally examined MnSOD and CuZnSOD mRNA and protein expression in these lung tumors and found no statistical differences from the adjacent normal lung tissue (**Supplemental Fig. 3**). Next to test the hypothesis that EcSOD repression in lung tumors is associated with a more methylated *EcSOD* promoter, we performed COBRA analysis followed by real-time PCR amplification to determine differential methylation status between the normal and tumor samples. As indicated the **Fig. 3D**, 8/10 (80%) of the tumor samples showed increases in the COBRA methylation index as determined by loss of signal due to TaqI enzyme's ability to restrict the TCGA site. It is interesting to note that the two samples without significant methylation changes had relatively higher levels of EcSOD mRNA expression. To more extensively examine the association between EcSOD expression and promoter methylation we performed a Sequenom MassArray analysis on four of the lowest EcSOD expressing tumors (T2-T5), an intermediate expressor (T1), all confirmed by histopathology to contain $\geq 70\%$ cancer cells, and normal lung (NL). Western blotting for EcSOD protein in lung tumors T1-T5 (**Fig. 4A**) showed decreased expression compared to normal lung. Four of tumors assessed (T2-T5) showed an 80% or greater reduction in EcSOD mRNA expression (**Fig. 4B**), while one (T1) was an intermediate expressor when compared to normal lung. **Fig. 4C** shows the heat map generated from the Sequenom analysis indicating the CpG sites (CpG 6-12, **Fig. 2A**) surrounding the Sp1/Sp3 site in the *EcSOD* promoter in T2-T5 had a statistically significantly higher percentage of cytosine methylation when compared to NL or the intermediate EcSOD expressing T1 sample. These data indicate that transcriptional silencing of EcSOD mRNA expression in lung adenocarcinoma cells both *in vitro* and *in vivo* is linked to aberrant cytosine methylation of the *EcSOD* promoter in the Sp1/Sp3 regulatory region. Raw CpG methylation percentages that correspond to the heat map are shown in **Supplemental Fig. 4**.

Loss of heterozygosity (LOH) at the *EcSOD* locus in clinical lung adenocarcinomas

Previous studies have identified the chromosomal location 4p15.3-4p15.1 as a frequent location for LOH in numerous cancers including; lung, breast, colorectal, bladder, etc. Of particular interest is the study by Shivapurkar, *et al.* which showed LOH of 4p15.3-4p15.1 in 25% of NSCLC (25). To determine whether *EcSOD*, located at 4p15.3-4p15.1, was an affected gene in this region of LOH, we performed a TaqMan Copy Number assay with a probe overlapping exon 2 of *EcSOD*. As shown in **Fig. 4D**, we found that 2/10 (20%) of the human adenocarcinomas examined exhibited LOH (T4, T8) as represented by loss of one copy of *EcSOD* when compared to normal lung tissue. In addition we showed that the EcSOD negative A549 cells showed LOH, whereas the EcSOD positive H1975 cells maintained two copies of the *EcSOD* locus. Both tumor specimens with LOH (T4, T8) as well as A549 cells displayed aberrant *EcSOD* promoter methylation (**Figs. 2B and 3D**). Together these results confirm and extend previous studies by identifying EcSOD as one of the genes affected by the loss of heterozygosity on chromosome 4p15.3-4p15.1 in lung tumors. More importantly, these results further implicate *EcSOD* as a tumor suppressor gene in lung cancer since it is a target of both LOH and epigenetic silencing.

Overexpression of catalytically active EcSOD in lung adenocarcinoma cells

Western blot analysis confirmed that neither A549 nor H1650 cells express EcSOD, but high levels of EcSOD protein could be detected in cell lysates and in culture media 48 hours after AdEcSOD infection (**Fig. 5A**). The activity gel shown in the bottom most panel indicates that EcSOD is catalytically active in culture media.

EcSOD significantly reduced the *in vitro* clonogenic fraction of A549 cells

We next performed clonogenic assays to determine the effect of EcSOD re-expression on the plating efficiency of lung cancer cells. Forced EcSOD re-expression significantly reduced the clonogenic fraction of A549 cells by 66% when compared to the AdEmpty infection (**Fig. 5B**). This result indicated that forced re-expression of EcSOD had an inhibitory effect on A549 cells, and suggests that loss of EcSOD may augment cell proliferation.

EcSOD inhibits the invasive potential of lung adenocarcinoma cells

The photomicrographs in **Fig. 5C** show representative fields from the Matrigel *in vitro* invasion assays for Control, AdEmpty and AdEcSOD infected cells and suggested that EcSOD inhibited Matrigel invasion. When quantified and compared to the AdEmpty infected cells, over-expression of EcSOD significantly reduced invasion by 65% and 72% in A549 and H1650 cells, respectively (**Fig. 5D**). These results clearly illustrate that the forced re-expression of EcSOD had a marked effect on the invasive potential of lung adenocarcinoma cells.

EcSOD attenuated heparanase expression and activity in lung adenocarcinoma cells by inhibiting heparanase promoter activity

The importance of heparanase in lung adenocarcinomas was recently shown by Cohen, *et al.* who reported that overexpression of heparanase correlated inversely with patient survival (26). We report here that EcSOD impaired the expression of heparanase by inhibiting its promoter activity in both A549 and H1650 cell lines. **Fig 6A** indicates that AdEcSOD over-expressing A549 and H1650 cells had >3-fold and 2-fold reductions in heparanase mRNA expression respectively when compared to AdEmpty-infected cells. Furthermore, heparanase promoter activity, as determined by a luciferase reporter assay, was decreased by >80% in EcSOD over-expressing cells (**Fig. 6B**). Next we overexpressed the superoxide scavengers CuZnSOD and MnSOD in A549 cells and report here that only EcSOD had a significant effect on heparanase mRNA expression (**Fig. 6C**).

EcSOD downregulated syndecan-1 in lung adenocarcinoma cells

EcSOD has been shown to bind to the heparin binding domain of the HSPG syndecan-1 and protect it from oxidative cleavage in the lung (27). In addition, it has been shown that overexpression of heparanase leads to increased transcriptional expression and shedding of syndecan-1 (28). Because we saw a reduction in heparanase expression in lung adenocarcinoma cells after re-introduction of EcSOD we hypothesized that the steady state levels of syndecan-1 mRNA would also be reduced. Our results in both A549 and H1650 cells infected with AdEcSOD indeed showed decreased steady state levels of syndecan-1 mRNA by 22% and 36% respectively compared to AdEmpty controls (**Fig. 6D**).

EcSOD decreases the basal NF- κ B activity in lung adenocarcinoma cells

NF- κ B activation has been shown to correlate with an increase in heparanase expression and is frequently overexpressed in human lung cancer (29-30). Blockage of NF- κ B down-regulates heparanase expression in a murine lung alveolar carcinoma cell line, and recently NF- κ B has been shown to be required for the development of a mouse model of lung adenocarcinoma (31-32). So to test if EcSOD could affect the activity of the redox sensitive NF- κ B we used an adenovirus construct with an NF- κ B responsive luciferase reporter to assess NF- κ B transcriptional activity after forced re-expression of EcSOD. Our results show that basal NF- κ B activity was decreased by 47% and 55% in A549 and H1650 cells (**Fig. 6E**).

Downregulation of EcSOD is an early event in several lung cancer types

To determine at what stage during lung carcinogenesis, and whether downregulation of EcSOD is a common event in differing lung cancer tumor types, we performed a TissueScan Lung cancer cDNA Array (Origene, Inc.), which included 40 tumors ranging from Stage I through Stage IV and 8 normal lung tissue controls. **Fig. 7A** shows the percent (%) mRNA expression of EcSOD by stage and tumor type. As early as Stage I, EcSOD expression is significantly reduced ($P < .001$) and there is a consistent reduction by Stage IV, with statistical significance of $P < .05$ between Stage I and Stage IV. These data clearly show that in 38/40 additional human lung tumors EcSOD is a target of repression, and that this occurs not only in adenocarcinoma, but also in several other types of lung tumors. This extends the significance of our findings beyond adenocarcinoma to other lung tumor types as well. Moreover, loss of EcSOD appears to be an early event in lung carcinogenesis as expression is frequently lost in Stage I disease.

Discussion

The purpose of this study was to investigate the expression, regulation and function of EcSOD in lung cancer. The role of EcSOD in normal lung development and several lung pathophysiologies including pulmonary fibrosis and COPD has been well characterized, but comparably few studies have examined its role in lung carcinogenesis.

The importance of scavenging ROS in the lung by superoxide dismutase is critical because of its unique exposure to oxidant stress. Besides exposure to ambient air, the lung is also exposed to numerous environmental irritants (6, 12). The primary feature of most pulmonary diseases is inflammation and activation of inflammatory cells as a consequence of the production of reactive oxygen species (33). This inflammatory response to oxidants activates transcription factors including NF- κ B that are associated with induction of antioxidant enzymes in the lung (34). Therefore, the response and expression of EcSOD, which comprises the only source of extracellular SOD activity in the lung (including >70% of the SOD activity in pulmonary and systemic vessels)(3), is critical to the better understanding of the pathology of lung cancer.

To accomplish this we examined EcSOD expression in lung adenocarcinoma cell lines and lung adenocarcinoma tumors compared to their normal counterparts and found significant decreases in both EcSOD mRNA and protein expression (Fig. 3A,B,C and Fig. 4B, A). Whereas EcSOD expression was affected, neither MnSOD nor CuZnSOD were altered compared to normal lung at either the mRNA or protein level. This reduction of only EcSOD in lung cancer cells both *in vitro* and *in vivo* was striking because of the lungs' reliance on EcSOD to scavenge ROS in the extracellular compartment. This has been best demonstrated in EcSOD knockout mice that show enhanced lung injury to environmental insults (12). In contrast, transgenic mice that overexpress EcSOD have been shown to prevent lung inflammation in part due to the ability of EcSOD to bind to the negatively charged constituents in the extracellular matrix and prevent oxidative degradation of hyaluronan, Type I collagen and heparin sulfate (10, 35). Moreover, mice with a conditional reduction of EcSOD by >50% in the lung showed severe lung injury and death by 7 days (36). These results highlight the importance of EcSOD in maintenance of normal lung physiology, and together with our findings that EcSOD expression was markedly decreased in lung cancer could extend EcSOD's significance to a role in lung carcinogenesis.

To better understand the mechanisms involved in the downregulation of EcSOD in lung adenocarcinomas we examined whether epigenetic dysregulation by aberrant DNA promoter methylation of *EcSOD* could be responsible for the marked reduction of EcSOD expression observed both *in vitro* and *in vivo*. In each case where EcSOD mRNA expression was

significantly decreased we found aberrant DNA methylation of the 5' end of the *EcSOD* gene (Fig. 2, 3D, 4B). We noted aberrant methylation surrounding the Sp1/Sp3 transcription factor binding site in the *EcSOD* promoter. Comparable methylation of a Sp1/Sp3 site and its adjacent regions has previously been shown to lead to transcriptional repression of the p21 (CIP1) gene (37). This can occur through direct inhibition of the transcription factors or by binding of methyl-binding proteins which recruit histone deacetylases (HDACs).

In addition to the Sp1/Sp3 binding site, this region of the *EcSOD* promoter also contains an aryl-hydrocarbon receptor-xenobiotic response element (AhR-XRE) (Fig. 2A) which has been predicted to play a role in *EcSOD* regulation (38). Recently, Ganguly and colleagues showed that a SNP creating a CpG to TpG change of this AhR-XRE site in the *EcSOD* promoter was associated with diminished lung function in children (39). While we observed neither this SNP nor any other genetic mutations during our extensive sequencing of the *EcSOD* promoter in the cell lines examined here, it has previously been shown that methylation of the CpG in the core AhR-XRE binding site can inhibit binding of this transcription factor (40). This is of particular interest in lung cancer because cigarette smoke contains an abundant quantity of AhR ligands which have been shown to induce cytochrome P450 (Cyp) leading to a pro-inflammatory response by cytokines, chemokines and ultimately ROS production. Our findings illustrated that in lung cancer cells this AhR-XRE cis-element becomes aberrantly methylated in the *EcSOD* promoter, which may prevent the induction of *EcSOD* by AhR ligands and inhibit *EcSOD* from compensating for the induction of cytochrome P450 and its downstream production of ROS.

Based on previous cytogenetic studies that show the 4p15 location on chromosome 4 is a hotspot for deletion in many carcinomas, we examined whether LOH occurred at the *EcSOD* allele in lung adenocarcinoma tumors. We found LOH of *EcSOD* (20% in lung adenocarcinoma tumors), consistent with the published literature at the location of 4p15.3-4p15.1 showing LOH in 25% of NSCLC tumors (25). This region of chromosome 4 also contains the previously described *Slit2* tumor suppressor gene, which resembles *EcSOD* inactivation by exhibiting both promoter DNA hypermethylation and allelic loss in lung and breast cancers (41). It is interesting to speculate that along with *Slit2*, *EcSOD* could be a potential tumor suppressor gene that is aberrantly affected by both genetic and epigenetic mechanisms in this region of chromosome 4 during carcinogenesis. Another interesting study confirmed the importance of this region of chromosome 4 in breast cancer development. The treatment of MCF10a cells with exogenous estrogen lead to a neoplastic and invasive phenotype only after the deletion of chromosome 4p15.3-16. This suggested to the authors that this region of chromosome 4 could be an early event that triggered a cascade that selected for a tumorigenic cell population (42).

The consequences of this loss of *EcSOD* in lung cancer cells may be highlighted by our forced overexpression of *EcSOD* that lead to a marked reduction in both clonogenic and invasive potential (Fig 5B, C), demonstrating that *EcSOD* could partially reverse their malignant phenotype. This partial reversion of our lung cancer cells by *EcSOD* could in part be attributed to the alteration of some benchmark factors associated with the malignant phenotype of lung cancer: heparanase, syndecan-1, and NF- κ B. Heparanase has been shown to be upregulated in all cancers examined to date including in lung cancer where it is associated with poor patient outcomes (26, 43). In this study we provide evidence that re-expression of *EcSOD* in lung cancer cells downregulated heparanase mRNA and activity by inhibiting heparanase promoter activity (Fig. 6A, B). However, overexpression of the two other superoxides dismutases the cytosolic CuZnSOD and mitochondrial MnSOD had no significant impact on heparanase expression (Fig. 6C). This exciting result adds to the novel role that *EcSOD* may be playing in protecting constituents of the lung extracellular matrix. As mentioned earlier, *EcSOD* has been shown in numerous studies to protect heparin

sulfate, collagen, and hyaluronan in the lung from oxidative fragmentation. Our new results indicate that EcSOD could be acting in a dual role to protect lung extracellular matrix integrity both by scavenging ROS and by inhibiting the cleavage of HS through downregulation of heparanase. In addition to its role in cleaving HS, heparanase has been shown to induce the transcription of syndecan-1 (28, 44). The HSPG syndecan-1 acts as a storage depot for cytokines and growth factors and when cleaved by heparanase facilitates tumor growth, angiogenesis and metastasis. Moreover, high levels of syndecan-1 are associated with poor prognosis in lung cancer patients (45-46). Figure 6E shows that overexpression of EcSOD did moderately downregulate syndecan-1 expression. This downregulation is very consistent with a recent study showing that siRNA knockdown of heparanase in myeloma cells showed a 38% reduction in the level of syndecan-1 mRNA expression (44). We believe that EcSOD is exerting its effects on heparanase and its downstream target syndecan-1, at least in part, by decreasing the transcriptional activity of NF- κ B (Fig. 6F).

Another important clinical question is at what stage is EcSOD expression downregulated, is it common to differing types of lung cancers and is it contributing to the initiation, metastasis, or is it a later carcinogenic event? In this manuscript we sought to determine at what stage and in what lung cancer types is EcSOD downregulated. We found that as early as Stage I EcSOD mRNA expression was significantly reduced with a continual reduction by Stage IV ($P < .001$), and was common in all lung tumor types. This stage I loss of EcSOD expression could lead to not only degradation of ECM structures compromising barrier function but also to the release of heparin bound growth stimulatory factors such as HB-EGF, VEGF, and FGF to their receptors thus promoting growth. We are currently conducting experiments to knock down EcSOD expression in normal human bronchial epithelial (NHBE) cells and examining ECM integrity, proliferation and possible cancer initiation.

What may be the implications of this silencing of EcSOD be for therapy of human lung cancer? First, pulmonary gene therapy could be utilized to reinforce EcSOD expression in the lung, though this strategy has obvious inherent difficulties. Perhaps a more tractable clinical approach would be the use of SOD mimetic drugs which have been under development and clinical testing as anti-inflammatory agents for some time and their use is beginning to appear promising. Finally, the absence of SOD activity in the extracellular space might create a more pro-oxidative tumor microenvironment that could be taken advantage of by emerging redox therapies such as high dose intravenous ascorbate which has shown promise against pancreatic cancer (47-48).

Taken together we have shown that both epigenetic silencing and genetic alterations affecting the expression of EcSOD are common event in lung cancer. Loss of EcSOD likely exerts a profound impact on the homeostasis of pulmonary extracellular matrix, and given its vital role in other pulmonary diseases, EcSOD may play an important but previously unrecognized role in human lung cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. James Crapo (National Jewish Medical and Research Center, Denver, CO) for providing antibodies to EcSOD and Anna G. Johns for expert technical assistance. This study was supported by NIH grants R01CA115438, R01CA073612, P30CA086862, R01 CA065662, 5P30DK054759 (sub # 9004, Cells and Tissues Core), Komen for the Cure grant KG080437 and SFRBM Research Mini-Fellowship. We dedicate this work to the

late Dr. Larry Oberley whose pioneering studies on SOD in cancer we honor, and to Rita Selle whose courage helped inspire this work.

References

1. Jung O, Marklund SL, Geiger H, Pedrazzini T, Busse R, Brandes RP. Extracellular superoxide dismutase is a major determinant of nitric oxide bioavailability: in vivo and ex vivo evidence from ecSOD-deficient mice. *Circ Res*. 2003; 93:622–9. [PubMed: 12933702]
2. Marklund SL. Extracellular superoxide dismutase in human tissues and human cell lines. *J Clin Invest*. 1984; 74:1398–403. [PubMed: 6541229]
3. Oury TD, Day BJ, Crapo JD. Extracellular superoxide dismutase in vessels and airways of humans and baboons. *Free Radic Biol Med*. 1996; 20:957–65. [PubMed: 8743981]
4. Arcaroli JJ, Hokanson JE, Abraham E, Geraci M, Murphy JR, Bowler RP, et al. Extracellular superoxide dismutase haplotypes are associated with acute lung injury and mortality. *Am J Respir Crit Care Med*. 2009; 179:105–12. [PubMed: 18948423]
5. Young RP, Hopkins R, Black PN, Eddy C, Wu L, Gamble GD, et al. Functional variants of antioxidant genes in smokers with COPD and in those with normal lung function. *Thorax*. 2006; 61:394–9. [PubMed: 16467073]
6. Fattman CL, Chang LY, Termin TA, Petersen L, Enghild JJ, Oury TD. Enhanced bleomycin-induced pulmonary damage in mice lacking extracellular superoxide dismutase. *Free Radic Biol Med*. 2003; 35:763–71. [PubMed: 14583340]
7. Carlsson LM, Jonsson J, Edlund T, Marklund SL. Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. *Proc Natl Acad Sci U S A*. 1995; 92:6264–8. [PubMed: 7603981]
8. Bowler RP, Nicks M, Warnick K, Crapo JD. Role of extracellular superoxide dismutase in bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2002; 282:L719–26. [PubMed: 11880297]
9. Sandstrom J, Carlsson L, Marklund SL, Edlund T. The heparin-binding domain of extracellular superoxide dismutase C and formation of variants with reduced heparin affinity. *J Biol Chem*. 1992; 267:18205–9. [PubMed: 1517248]
10. Kliment CR, Tobolewski JM, Manni ML, Tan RJ, Enghild J, Oury TD. Extracellular superoxide dismutase protects against matrix degradation of heparan sulfate in the lung. *Antioxid Redox Signal*. 2008; 10:261–8. [PubMed: 17961072]
11. Gao F, Koenitzer JR, Tobolewski JM, Jiang D, Liang J, Noble PW, et al. Extracellular superoxide dismutase inhibits inflammation by preventing oxidative fragmentation of hyaluronan. *J Biol Chem*. 2008; 283:6058–66. [PubMed: 18165226]
12. Fattman CL, Tan RJ, Tobolewski JM, Oury TD. Increased sensitivity to asbestos-induced lung injury in mice lacking extracellular superoxide dismutase. *Free Radic Biol Med*. 2006; 40:601–7. [PubMed: 16458190]
13. Teoh ML, Fitzgerald MP, Oberley LW, Domann FE. Overexpression of extracellular superoxide dismutase attenuates heparanase expression and inhibits breast carcinoma cell growth and invasion. *Cancer Res*. 2009; 69:6355–63. [PubMed: 19602586]
14. Kinnula VL, Hodgson UA, Lakari EK, Tan RJ, Sormunen RT, Soini YM, et al. Extracellular superoxide dismutase has a highly specific localization in idiopathic pulmonary fibrosis/usual interstitial pneumonia. *Histopathology*. 2006; 49:66–74. [PubMed: 16842247]
15. Dahl M, Bowler RP, Juul K, Crapo JD, Levy S, Nordestgaard BG. Superoxide dismutase 3 polymorphism associated with reduced lung function in two large populations. *Am J Respir Crit Care Med*. 2008; 178:906–12. [PubMed: 18703790]
16. Svensk AM, Soini Y, Paakko P, Hiravikoski P, Kinnula VL. Differential expression of superoxide dismutases in lung cancer. *Am J Clin Pathol*. 2004; 122:395–404. [PubMed: 15362370]
17. Zelko IN, Mueller MR, Folz RJ. Transcription factors sp1 and sp3 regulate expression of human extracellular superoxide dismutase in lung fibroblasts. *Am J Respir Cell Mol Biol*. 2008; 39:243–51. [PubMed: 18314536]
18. Stern LF, Chapman NH, Wijsman EM, Altherr MR, Rosen DR. Assignment of SOD3 to human chromosome band 4p15.3-->p15.1 with somatic cell and radiation hybrid mapping, linkage

- mapping, and fluorescent in-situ hybridization. *Cytogenet Genome Res.* 2003; 101:178. [PubMed: 14619883]
19. Shivapurkar N, Virmani AK, Wistuba II, Milchgrub S, Mackay B, Minna JD, et al. Deletions of chromosome 4 at multiple sites are frequent in malignant mesothelioma and small cell lung carcinoma. *Clinical Cancer Research.* 1999; 5:17–23. [PubMed: 9918198]
  20. Dallol A, Da Silva NF, Viacava P, Minna JD, Bieche I, Maher ER, et al. SLIT2, a human homologue of the *Drosophila* Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. *Cancer Research.* 2002; 62:5874–80. [PubMed: 12384551]
  21. Shivapurkar N, Sood S, Wistuba II, Virmani AK, Maitra A, Milchgrub S, et al. Multiple regions of chromosome 4 demonstrating allelic losses in breast carcinomas. *Cancer Research.* 1999; 59:3576–80. [PubMed: 10446964]
  22. Chen JH, Stoltz DA, Karp PH, Ernst SE, Pezzulo AA, Moninger TO, et al. Loss of anion transport without increased sodium absorption characterizes newborn porcine cystic fibrosis airway epithelia. *Cell.* 2010; 143:911–23. [PubMed: 21145458]
  23. Rose SL, Fitzgerald MP, White NO, Hitchler MJ, Futscher BW, De Geest K, et al. Epigenetic regulation of maspin expression in human ovarian carcinoma cells. *Gynecol Oncol.* 2006; 102:319–24. [PubMed: 16457875]
  24. Teoh ML, Sun W, Smith BJ, Oberley LW, Cullen JJ. Modulation of reactive oxygen species in pancreatic cancer. *Clin Cancer Res.* 2007; 13:7441–50. [PubMed: 18094428]
  25. Shivapurkar N, Virmani AK, Wistuba II, Milchgrub S, Mackay B, Minna JD, et al. Deletions of chromosome 4 at multiple sites are frequent in malignant mesothelioma and small cell lung carcinoma. *Clin Cancer Res.* 1999; 5:17–23. [PubMed: 9918198]
  26. Cohen E, Doweck I, Naroditsky I, Ben-Izhak O, Kremer R, Best LA, et al. Heparanase is overexpressed in lung cancer and correlates inversely with patient survival. *Cancer.* 2008; 113:1004–11. [PubMed: 18618498]
  27. Kliment CR, Englert JM, Gochuico BR, Yu G, Kaminski N, Rosas I, et al. Oxidative stress alters syndecan-1 distribution in lungs with pulmonary fibrosis. *J Biol Chem.* 2009; 284:3537–45. [PubMed: 19073610]
  28. Yang Y, Macleod V, Miao HQ, Theus A, Zhan F, Shaughnessy JD Jr. et al. Heparanase enhances syndecan-1 shedding: a novel mechanism for stimulation of tumor growth and metastasis. *J Biol Chem.* 2007; 282:13326–33. [PubMed: 17347152]
  29. Wu W, Pan C, Meng K, Zhao L, Du L, Liu Q, et al. Hypoxia activates heparanase expression in an NF-kappaB dependent manner. *Oncol Rep.* 2010; 23:255–61. [PubMed: 19956890]
  30. Tang X, Liu D, Shishodia S, Ozburn N, Behrens C, Lee JJ, et al. Nuclear factor-kappaB (NF-kappaB) is frequently expressed in lung cancer and preneoplastic lesions. *Cancer.* 2006; 107:2637–46. [PubMed: 17078054]
  31. Andela VB, Schwarz EM, Puzas JE, O'Keefe RJ, Rosier RN. Tumor metastasis and the reciprocal regulation of prometastatic and antimetastatic factors by nuclear factor kappaB. *Cancer Res.* 2000; 60:6557–62. [PubMed: 11118032]
  32. Meylan E, Dooley AL, Feldser DM, Shen L, Turk E, Ouyang C, et al. Requirement for NF-kappaB signalling in a mouse model of lung adenocarcinoma. *Nature.* 2009; 462:104–7. [PubMed: 19847165]
  33. Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. *Am J Respir Crit Care Med.* 2003; 167:1600–19. [PubMed: 12796054]
  34. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279:L1005–28. [PubMed: 11076791]
  35. Petersen SV, Oury TD, Ostergaard L, Valnickova Z, Wegrzyn J, Thogersen IB, et al. Extracellular superoxide dismutase (EC-SOD) binds to type I collagen and protects against oxidative fragmentation. *J Biol Chem.* 2004; 279:13705–10. [PubMed: 14736885]
  36. Gongora MC, Lob HE, Landmesser U, Guzik TJ, Martin WD, Ozumi K, et al. Loss of extracellular superoxide dismutase leads to acute lung damage in the presence of ambient air: a potential mechanism underlying adult respiratory distress syndrome. *Am J Pathol.* 2008; 173:915–26. [PubMed: 18787098]

37. Zhu WG, Srinivasan K, Dai Z, Duan W, Druhan LJ, Ding H, et al. Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter. *Mol Cell Biol.* 2003; 23:4056–65. [PubMed: 12773551]
38. Hanlon PR, Zheng W, Ko AY, Jefcoate CR. Identification of novel TCDD-regulated genes by microarray analysis. *Toxicol Appl Pharmacol.* 2005; 202:215–28. [PubMed: 15667827]
39. Ganguly K, Depner M, Fattman C, Bein K, Oury TD, Wesselkamper SC, et al. Superoxide dismutase 3, extracellular (SOD3) variants and lung function. *Physiol Genomics.* 2009; 37:260–7. [PubMed: 19318538]
40. Kikuchi Y, Ohsawa S, Mimura J, Ema M, Takasaki C, Sogawa K, et al. Heterodimers of bHLH-PAS protein fragments derived from AhR, AhRR, and Arnt prepared by co-expression in *Escherichia coli*: characterization of their DNA binding activity and preparation of a DNA complex. *J Biochem.* 2003; 134:83–90. [PubMed: 12944374]
41. Dallol A, Da Silva NF, Viacava P, Minna JD, Bieche I, Maher ER, et al. SLIT2, a human homologue of the *Drosophila* Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. *Cancer Res.* 2002; 62:5874–80. [PubMed: 12384551]
42. Russo J, Fernandez SV, Russo PA, Fernbaugh R, Sheriff FS, Lareef HM, et al. 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *FASEB J.* 2006; 20:1622–34. [PubMed: 16873885]
43. Vlodavsky I, Elkin M, Abboud-Jarrous G, Levi-Adam F, Fuks L, Shafat I, et al. Heparanase: one molecule with multiple functions in cancer progression. *Connect Tissue Res.* 2008; 49:207–10. [PubMed: 18661344]
44. Mahtouk K, Hose D, Raynaud P, Hundemer M, Jourdan M, Jourdan E, et al. Heparanase influences expression and shedding of syndecan-1, and its expression by the bone marrow environment is a bad prognostic factor in multiple myeloma. *Blood.* 2007; 109:4914–23. [PubMed: 17339423]
45. Joensuu H, Anttonen A, Eriksson M, Makitaro R, Alfthan H, Kinnula V, et al. Soluble syndecan-1 and serum basic fibroblast growth factor are new prognostic factors in lung cancer. *Cancer Res.* 2002; 62:5210–7. [PubMed: 12234986]
46. Papay J, Krenacs T, Moldvay J, Stelkovics E, Furak J, Molnar B, et al. Immunophenotypic profiling of nonsmall cell lung cancer progression using the tissue microarray approach. *Appl Immunohistochem Mol Morphol.* 2007; 15:19–30. [PubMed: 17536303]
47. Du J, Martin SM, Levine M, Wagner BA, Buettner GR, Wang SH, et al. Mechanisms of ascorbate-induced cytotoxicity in pancreatic cancer. *Clin Cancer Res.* 2010; 16:509–20. [PubMed: 20068072]
48. Espey MG, Chen P, Chalmers B, Drisko J, Sun AY, Levine M, et al. Pharmacologic ascorbate synergizes with gemcitabine in preclinical models of pancreatic cancer. *Free Radic Biol Med.* 2011; 50:1610–9. [PubMed: 21402145]

Figure 1. EcSOD expression is absent in human lung adenocarcinoma cells in association with an inaccessible chromatin structure, and can be reactivated by inhibiting DNA methyltransferase

A. EcSOD protein was detected by western blot analysis in two normal (HAE, HMEC) cell lines and three lung adenocarcinoma cell lines (H1975, A549, H1650). **B.** The levels of EcSOD mRNA were measured by RT-PCR with 18S used as a loading control. **C.** Three lung adenocarcinoma cell lines were queried for sensitivity to DNase I restriction then accessed by real-time PCR with primers surrounding the Sp1/Sp3 binding site (Fig. 2A). Accessibility index was determined as follows: Accessibility index = $2^{((Ct \text{ Dnase treated}) - (Ct \text{ Uncut}))}$. GAPDH was used as a positive control for accessibility. See Supplemental Fig. 4. *Error Bars*, SD of three assays. Statistical analysis was performed using one-way ANOVA followed by post-hoc Tukey's test: *, $P < .05$ versus H1975 cells. **D.** The EcSOD-negative H1650 cells were treated with the DNA methyltransferase inhibitor 5-Aza-dC at 5 μ M for 5 days and analyzed by RT-PCR for EcSOD expression. PCR products were visualized on 2% agarose gels with ethidium bromide staining.

Figure 2. Absence of EcSOD expression in lung cancer cells is associated with aberrant methylation of the 5' end of the *EcSOD* gene

A. The region of the *EcSOD* promoter examined within this study spanned from -550 to +100 of the putative transcription start site (+1) or bp 24796689-24797348 of the Feb. 2009 assembly of the UCSC genome browser. This region contained 18 CpG sites represented by black vertical slash marks (I) and the SP1/SP3 binding site location (-212 to -198 bp). Also displayed is the location of Sequenom MassArray Analysis performed in Fig. 4, and the primer locations for chromatin accessibility study in Fig. 1C (CA1, CA2). **B.** The methylation status of the 18 CpG sites in the *EcSOD* promoter was determined by bisulfite genomic sequencing from three *EcSOD*-positive (left column) and three *EcSOD*-negative cell lines (right column). Each row of circles represents the cytosine methylation pattern obtained from an individual clone of the bisulfite treated, PCR amplified and sequenced *EcSOD* promoter. Each row represents 1 of 5 clones picked. The position of each CpG is labeled 1-18 as represented in Fig. 2A. Open circles represent unmethylated CpGs; filled

circles indicate methylated CpG. The highlighted gray box corresponds to the 6 CpG's surrounding the Sp1/Sp3 site.

Figure 3. EcSOD mRNA expression is significantly lower in primary human lung adenocarcinomas compared to normal human lung tissue, and this decrease is associated with significantly increased promoter methylation

A. Total mRNA was isolated from frozen sections of ten different primary human lung adenocarcinoma tumors. Quantitative real-time PCR was performed using *EcSOD* specific TaqMan primer-probe. Relative *EcSOD* mRNA expression for each tumor sample is represented as [(average ΔCt of $n=5$ normal lung tissue samples) – (ΔCt tumor sample)]. Samples were normalized to 18S. Dotted lines represent ± 1 S.D. for normal lung tissue *EcSOD* mRNA expression. **B.** Box plot depicting *EcSOD* mRNA expression from samples shown in A. P value is based on ANOVA between 5 normal samples and 10 cancer samples. **C.** Direct *EcSOD* mRNA expression comparison between four matched pairs of normal:tumor samples. **D.** Real-time PCR amplification results from a COBRA analysis followed by amplification using primers spanning the TAQI site in the *EcSOD* promoter. The COBRA methylation index was calculated as follows: $2^{[(Ct \text{ TaqI treated}) - (Ct \text{ Uncut})]}$. Error bars, S.D. of three assays, a two-tailed Student's t -test was performed: *, $P < 0.05$ versus NL.

Figure 4. Clinical lung adenocarcinomas display decreased EcSOD protein and mRNA expression in association with frequent aberrant EcSOD promoter methylation and occasional LOH

A. Western blot for EcSOD in the top panel with beta-actin loading control lower panel. NL represents normal lung tissue while LT1-LT5 are lung adenocarcinoma tumor samples. **B.** Expression of EcSOD mRNA as determined by quantitative real-time RT-PCR. The x-axis represents the clinical sample labels for both panels A & B. NL represents normal lung tissue; LT1-LT5 represents lung tumor 1 through 5. Each value is shown as a relative value compared to NL and normalized to 18S transcription level. *Error bars*, S.D. of three assays, a two-tailed Student's *t*-test was performed: *, $P < 0.05$ versus NL. **C.** Heat map depicting

the percent methylation of CpG sites 6-12 (right y-axis) of the EcSOD promoter in NL and LT1-LT5. The level of methylation of individual CpG sites within the Sequenom MassArray analysis (see Fig. 2A for location) is displayed with the lowest methylation in light yellow and highest methylation in dark blue according to the legend at the left. **D.** Results of TaqMan copy number variation assay in 5 normal human lung tissue samples, 10 lung adenocarcinoma tumors, and several lung cancer cell lines. The y-axis represents gene copy number and the x-axis represents the clinical samples. *Error bars*, S.D. of each sample run in triplicate.

Figure 5. Forced re-expression of EcSOD reduced the clonogenic capacity and invasive potential of human lung cancer cells

A. A western blot after adenovirus transduction of EcSOD in A549 and H1650 cells shows robust expression of EcSOD in both cell lysates and in culture media. The bottom panel is an activity gel and shows that EcSOD was secreted and was catalytically active in AdEcSOD infected A549 cells. AdEmpty infection did not induce EcSOD expression in either cell line. **B.** Clonogenic survival assay after forced EcSOD re-expression in A549 cells compared to control or AdEmpty infected cells. **C.** Representative photographs of cells that invaded through Matrigel membranes. **D.** Invasion through Matrigel was assessed after forced EcSOD re-expression in both A549 and H1650 cells and compared to control and AdEmpty infected cells. *Error Bars*, S.D. of three experiments; statistical analysis was a one-way ANOVA followed by post-hoc Tukey's test, *, $P < .05$ versus AdEmpty.

Figure 6. EcSOD is unique among the superoxide dismutases in decreasing heparanase mRNA and activity and results in reduced syndecan-1 mRNA in part through inhibition of NF-κB
A. Quantitative real-time RT-PCR analysis in A549 and H1650 lung cancer cells after forced re-expression of EcSOD and compared to control and AdEmpty infection. **B.** Heparanase promoter activity was evaluated using a luciferase reporter assay. **C.** Quantitative real-time RT-PCR analysis for heparanase in A549 cells after forced re-expression of CuZnSOD, MnSOD and EcSOD. **D.** Measurement by quantitative real-time RT-PCR analysis for syndecan-1 mRNA expression in A549 and H1650 cells after forced re-expression of EcSOD. **E.** NF-κB transcriptional activity was assessed by co-infection with AdEcSOD, AdEmpty and an AdNF-κB-Luc (adenovirus construct for NF-κB transcriptional activity that contains a luciferase reporter), Error bars, S.D. of three assays, *, P < 0.05 versus AdEmpty infection.

Figure 7. EcSOD mRNA is significantly reduced by Stage I in several lung cancer tumor types
A. The EcSOD mRNA expression was measured in 8 normal lung tissues versus 40 lung tumors by quantitative real-time PCR. The percent expression of EcSOD compared to normal was plotted by stage and tumor type. A significant reduction of $P < .001$ was observed from normal to all stages. **B.** Proposed model illustrating EcSOD silencing by promoter methylation, LOH and closed chromatin structure that results in an increase in ROS leading to the induction of NF-κB and heparanase consistent with promoting the malignant phenotype in lung cancer cells.