Genetic and Epigenetic Inactivation of Extracellular Superoxide Dismutase Promotes an Invasive Phenotype in Human Lung Cancer by Disrupting ECM Homeostasis

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Abstract
Extracellular superoxide dismutase (EcSOD) is an important superoxide scavenger in the lung in which 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 with 0/5 normal lung samples. In addition, 20% of the tumors showed loss of heterozygosity (LOH) of EcSOD. Reexpression 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-κB. 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. Mol Cancer Res; 10(1); 1–12. ©2011 AACR.

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; ref. 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 chronic obstructive pulmonary disease (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 with 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 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 (5 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 and colleagues reported that although 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 reacquisition of EcSOD might attenuate its malignant phenotype.

In addition to DNA promoter methylation analysis, the status of allelic loss manifested as loss of heterozygosity (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 non-small cell lung carcinoma (NSCLC; refs. 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, reexpression 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-κB 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 (HAE) cells 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 American Type Culture Collection (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 4 matched tumors: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 reverse transcription-PCR (RT-PCR) reactions were carried out with primers listed in Supplementary Table S1, 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 Supplementary Table S1.

Sodium bisulfite sequencing

Sodium bisulfite sequencing for EcSOD was carried out 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 to 24797348 of the Feb. 2009 assembly of the UCSC genome browser. Primers used are listed in Supplementary Table S1.

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, SnaBl, 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 Supplementary Table S1.

**DNA methylation by Sequenom MassArray analysis**
DNA methylation analysis was carried out using Sequenom MassARRAY according to manufacturer’s protocol. Primers used are shown in Supplementary Table S1.

**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 carried out 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 48 hours.

**Clonogenic assays**
After 48 hours 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 carried out 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 carried out 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 carried out using MYSTAT 12, for some experiments a 1-way ANOVA followed by post-hoc Tukey’s test was used for statistical difference between means. Statistical analyses were also assessed using a 2-tailed Student *t* test.

**Results**

EcSOD gene expression is silent in human lung adenocarcinoma cell lines, but can be reactivated by 5-Aza-dC treatment
Compared with normal 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, whereas in contrast A549, H1650, and HMEC cells had undetectable levels of EcSOD mRNA expression. These results indicate that the downregulation of EcSOD expression is occurring at the level of transcription. To examine whether the *EcSOD* gene promoter in nonexpressing 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 for A549 and H1650 cells were determined to be 5-fold and 3-fold less accessible, respectively, to nuclease digestion when compared with 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 whether cytosine methylation of *EcSOD* promoter region is associated with EcSOD transcriptional silencing, we conducted 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-
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 10 (100%) of the human lung adenocarcinoma tumors initially examined showed decreased mRNA expression of EcSOD when compared with the average range of expression in 5 normal lung samples (dotted lines). In Fig. 3B, the box-plot shows the significant difference (P < 0.001) 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 with 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 (Supplementary Fig. S3). Next to test the hypothesis that EcSOD repression in lung tumors is associated with a more methylated EcSOD promoter, we conducted COBRA analysis followed by real-time PCR amplification to determine differential methylation status between the normal and tumor samples. As indicated in 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 2 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 conducted a Sequenom MassArray analysis on 4 of the lowest EcSOD expressing tumors (T2–T5), an intermediate expressor (T1), all confirmed by histopathology to contain 70% or more cancer cells, and normal lung (NL). Western blotting for EcSOD protein in lung tumors T1–T5 (Fig. 4A) showed decreased expression compared with normal lung. Four of tumors assessed (T2–T5) showed an 80% or greater reduction in EcSOD mRNA expression (Fig. 4B), whereas one (T1) was an intermediate expressor when compared with normal lung. Figure 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 with 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 Supplementary Fig. S4.

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 and colleagues, 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 conducted 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 with normal lung tissue. In addition we showed that the EcSOD negative A549 cells showed LOH, whereas the EcSOD positive H1975 cells maintained 2 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 Figs. 3D). Together these results confirm and extend previous studies by identifying EcSOD as one of the genes affected by the LOH on chromosome 4p15.3-4p15.1 in lung tumors. More importantly, these results further implicate EcSOD as a tumor suppressor gene in lung cancer because 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 conducted clonogenic assays to determine the effect of EcSOD reexpression on the plating efficiency of lung cancer cells. Forced EcSOD reexpression significantly reduced the clonogenic fraction of A549 cells by 66% when compared with the AdEmpty infection (Fig. 5B). This result indicated that forced reexpression 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 with the AdEmpty-infected cells, overexpression of EcSOD significantly reduced invasion by 65% and 72% in A549 and H1650 cells, respectively (Fig. 5D). These
results clearly illustrate that the forced reexpression 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 and colleagues, 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. Figure 6A indicates that AdEcSOD overexpressing A549 and H1650 cells had more than 3-fold and 2-fold reductions in heparanase mRNA expression, respectively when compared with AdEmpty-infected cells. Furthermore, heparanase promoter activity, as determined by a luciferase reporter assay, was decreased by more than 80% in EcSOD overexpressing cells (Fig. 6B). Next, we over-expressed 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 reintroduction 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 with AdEmpty controls (Fig. 6D).
EcSOD decreases the basal NF-κB activity in lung adenocarcinoma cells

NF-κB 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-κB downregulates heparanase expression in a murine lung alveolar carcinoma cell line, and recently NF-κB has been shown to be required for the development of a mouse model of lung adenocarcinoma (31, 32). So to test whether EcSOD could affect the activity of the redox sensitive NF-κB, we used an adenovirus construct with an NF-κB responsive luciferase reporter to assess NF-κB transcriptional activity after forced reexpression of EcSOD. Our results show that basal NF-κB 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 conducted 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. Figure 7A shows the percent (%) mRNA expression of EcSOD by stage and tumor type. As early as stage I, EcSOD expression is significantly reduced (P < 0.001), and there is a consistent reduction by stage IV, with statistical significance of P < 0.05 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 seems 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-κB that are associated with induction of antioxidant enzymes in the lung (34). Therefore, the response and expression of EcSOD, which
The forced reexpression 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 reexpression in A549 cells compared with control or AdEmpty infected cells. C, representative photographs of cells that invaded through Matrigel membranes. D, invasion through Matrigel was assessed after forced EcSOD reexpression in both A549 and H1650 cells and compared with control and AdEmpty-infected cells. Error bars, SD of 3 experiments; statistical analysis was a 1-way ANOVA followed by post-hoc Tukey’s test. *, P < 0.05 versus AdEmpty.

Comprising the only source of extracellular SOD activity in the lung (including >70% of the SOD activity in pulmonary and systemic vessels; ref. 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 with their normal counterparts and found significant decreases in both EcSOD mRNA expression and protein expression (Figs. 3A–C and 4A and B). Whereas EcSOD expression was affected, neither MnSOD nor CuZnSOD were altered compared with 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 lung’s reliance on EcSOD to scavenge ROS in the extracellular compartment. This has been best shown 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 more than 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 (Figs. 2, 3D, and 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 (HDAC).

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). Although 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 proinflammatory 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.

On the basis of 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 and C), showing 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-κB. Heparanase has been shown to be upregulated in all cancers examined to date including in lung cancer in which it is associated with poor patient outcomes (26, 43). In this study, we provide evidence that reexpression of EcSOD in lung cancer cells downregulated heparanase mRNA and activity by inhibiting heparanase promoter activity (Fig. 6A and B). However, overexpression of the 2 other superoxide 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-κB (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 article, 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 ($P < 0.001$), 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 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 antiinflammatory agents for some time and their use is beginning to seem promising. Finally, the absence of SOD activity in the extracellular space might create a more prooxidative 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.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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