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Original Contribution

Epigenetic silencing of *SOD2* by histone modifications in human breast cancer cells

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ABSTRACT

Many breast cancer cells typically exhibit lower expression of manganese superoxide dismutase (MnSOD) compared to the normal cells from which they arise. This decrease can often be attributed to a defect in the transcription of *SOD2*, the gene encoding MnSOD; however, the mechanism responsible for this change remains unclear. Here, we describe how altered histone modifications and a repressive chromatin structure constitute an epigenetic process to down regulate *SOD2* in human breast carcinoma cell lines. Utilizing chromatin immunoprecipitation (ChIP) we observed decreased levels of dimethyl H3K4 and acetylated H3K9 at key regulatory elements of the *SOD2* gene. Consistent with these results, we show that loss of these histone modifications creates a repressive chromatin structure at *SOD2*. Transcription factor ChIP experiments revealed that this repressive chromatin structure influences the binding of SP-1, AP-1, and NFκB to *SOD2* regulatory *cis*-elements *in vivo*. Lastly, we show that treatment with the histone deacetylase inhibitors trichostatin A and sodium butyrate can reactivate *SOD2* expression in breast cancer cell lines. Taken together, these results indicate that epigenetic silencing of *SOD2* could be facilitated by changes in histone modifications and represent one mechanism leading to the altered expression of MnSOD observed in many breast cancers.

Introduction

Tumor cells are characterized by aberrant production of reactive oxygen species and an atypical redox state [1]. This has led to the hypothesis that reactive oxygen species may be causally involved in carcinogenesis. Changes in free radical biology in tumor cells can be linked to the altered expression pattern of antioxidant enzymes like MnSOD. Manganese superoxide dismutase (MnSOD) is a mitochondrial matrix enzyme encoded by the nuclear gene *SOD2*. Cancers originating from diverse tissue types often exhibit decreased MnSOD activity compared to the tissues from which they arise. At the same time, forced MnSOD overexpression in tumor-derived cell lines attenuates their malignant phenotype and lowers their metastatic potential [2–6]. These observations suggest that MnSOD may function as a new type of tumor-suppressor gene [7].

Breast cancer cells often have decreased levels of MnSOD compared to their normal counterparts, and this is generally attributed to a transcriptional defect of *SOD2* [8]. *SOD2* is regulated by several transcriptional regulatory elements that contain binding sites for AP-2, EGR-1, AP-1 and SP-1, NFκB, and FOXO3a [9–14]. While the *cis*-regulatory elements bound by these transcription factors are not commonly deleted or mutated in breast cancer [15], their accessibility to binding is required for them to play an active role in *SOD2* transcription. The accessibility of DNA and transcriptional regulatory elements is actuated through epigenetic processes.

Epigenetic alterations are a means by which gene expression can be altered independent of changes in DNA sequence. In the past decade it has become abundantly clear that epigenetic changes play just as important a role as mutations in carcinogenesis. Cytosine methylation at CpG dinucleotides is an epigenetic process that can silence gene expression. Hypermethylation of CpG dinucleotides within the *SOD2* basal promoter has been correlated with reduced levels of MnSOD in multiple myeloma and pancreatic carcinoma [16–18]. Similar studies from our group have revealed that DNA methylation reduces MnSOD levels in immortalized fibroblasts and breast cancer cells [19,20]. However, this observation was not ubiquitous among all breast cancer cell lines with decreased expression of *SOD2*. For example, MB-231and T47D, two cell lines used in this study, are two examples of human breast carcinoma cell lines that have decreased MnSOD expression, but whose *SOD2* promoters are unmethylated (Supplemental Fig. 1 and data not shown). Here, we chose to investigate whether changes in the posttranslational modification of histone tails could facilitate the decreased levels of MnSOD in human breast cancer cell lines.

The posttranslational acetylation and methylation of lysine residues in histone tails are epigenetic processes that can regulate

Abbreviations: MnSOD, manganese superoxide dismutase; mRNA, messenger RNA, ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline.

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gene expression [21,22]. Reduced levels of H3 histones methylated at lysine-4 and acetylated at lysine-9 within gene regulatory elements are indicative of gene silencing [23]. Previous studies have shown that MnSOD levels directly correlate with the acetylation status of histones present at *SOD2* [10,24]. Collectively these studies define the potential role for epigenetic regulation of *SOD2*.

We hypothesized that decreased levels of MnSOD in human breast cancer cell lines may be due, at least in part, to altered histone modifications and condensed chromatin structure at *SOD2*. In this study we identified decreased MnSOD protein and mRNA in four breast cancer cell lines compared to the immortalized, nontumorigenic breast epithelial cell line MCF-10A. Chromatin immunoprecipitation assays determined that the decreased expression of MnSOD in breast cancer cell lines correlated with hypoacetylation of H3K9 and hypomethylation of H3K4 at seven transcriptional regulatory elements within *SOD2*. Chromatin accessibility assays revealed that the chromatin structure of these seven regions is inaccessible compared to MCF-10A. Transcription factor chromatin immunoprecipitation uncovered decreased binding of NFκB, AP-1, and SP-1 at *SOD2* in breast cancer cell lines. A causal role of histone hypoacetylation in repressing MnSOD levels was determined by activating *SOD2* transcription using histone deacetylase inhibitors. Combined, these results suggest that MnSOD expression can be repressed in breast cancer by hypoacetylation and hypomethylation of histones, thus forming a closed chromatin structure at *SOD2* that inhibits transcription factor function.

Methods and materials

Tissue culture and drug treatments

The cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and grown as previously described [20]. Cells were maintained at 37 C and 5% CO_2 during all experiments. MB-231, T47D, MCF7, and MCF7-5C cells were either left untreated or treated with 200 ng/ml of trichostatin A or sodium butyrate dissolved in DMSO.

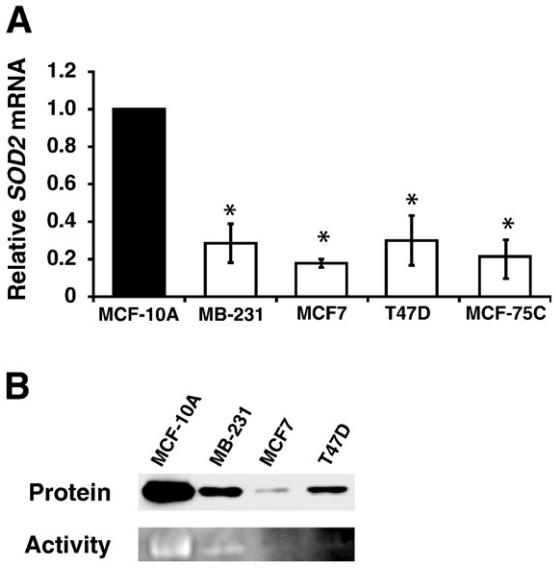
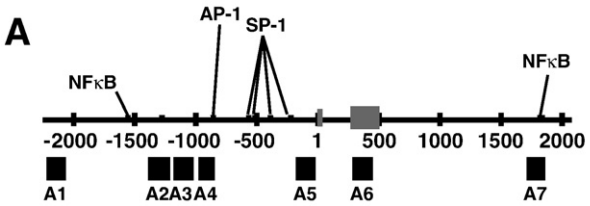


Fig. 1. Levels of MnSOD mRNA, protein, and activity are lower in breast cancer cell lines than immortalized breast epithelial cells. (A) Relative *SOD2* mRNA levels in breast cell lines determined by SYBR green real-time PCR. The steady-state levels of *SOD2* mRNA in the cell lines were normalized to 18S control, and then compared to MCF-10A using the $\Delta\Delta C_t$ method. Values shown are the average of three independent experiments with the standard error of mean (* $P<0.05$). (B) The levels of MnSOD protein and activity in the model cell lines used during this study.



B

Amp.	Forward	Reverse
A1	AGCTGATGACAGGTGTGATGTTG	TGAGAGTTTAAAGCGACTCCTAGGA
A2	AAGCCCAGCCCTTCCTGTT	GGTGCTGAACCGTTTCCGT
A3	CGCCAATGGCAGTGTAGATTT	CCTGCGCTGTCTTGTAGCCT
A4	TGACTGCCAGGGCTTAGTAGTGA	GAAATCCTTTGAGGTTAAGCAGCTT
A5	CTCCCCGCGCTTTCTTAAG	TCAACATGCTGCTAGTGCTGGT
A6	CGACCTGCCCTACGACTACG	CGCCTCCTGGTACTTCTCCTC
A7	GGAAAAGGCCCCGTGATTT	TCCTGGTGTCAGATGTTGCC

Fig. 2. Locations of the seven real-time PCR amplicons (A1–A7) used to analyze *SOD2* by chromatin immunoprecipitation (ChIP) and chromatin accessibility. Binding sites for AP-1, NFκB, and SP-1 within *SOD2* are indicated by black lines. (A) The seven real-time PCR amplicons used during histone and transcription factor ChIP as well as chromatin accessibility are represented by black boxes below the line. Exon 1 of the *SOD2* gene is designated by gray boxes. Numbering scheme was derived from build 17 of the human genome project. (B) Oligonucleotide primer sequences used to analyze the seven regions of *SOD2* during chromatin immunoprecipitation and chromatin accessibility experiments.

Quantitative reverse transcription real-time PCR analysis

Real-time PCR analysis of *SOD2* expression was carried out as previously described [20]. Analysis of SOD2 transcript levels was determined using 2X SYBR real-time master mix (Applied Biosystems) and primers specific to *SOD2*, or the control gene 18S. SYBR green fluorescence was then monitored using an ABI 7000 Sequence

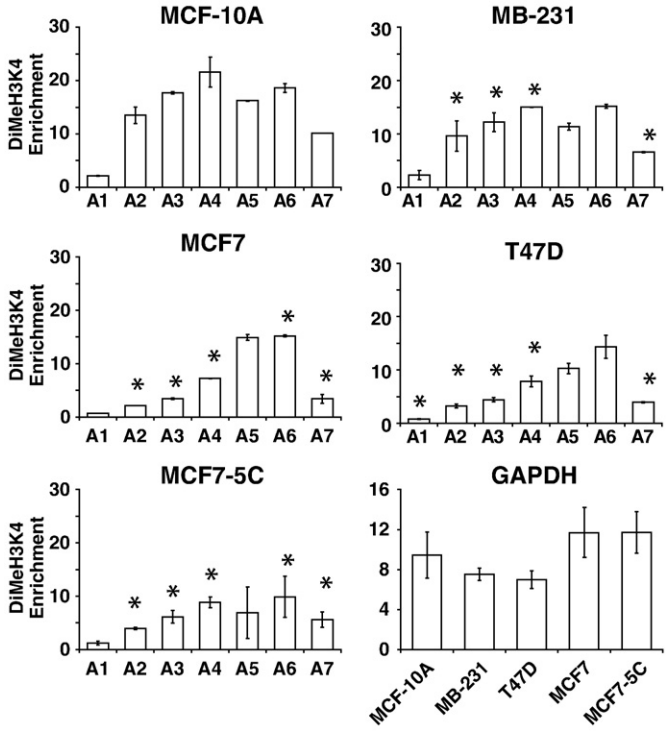


Fig. 3. Breast cancer cells have decreased levels of dimethyl H3K4 at *SOD2* compared to immortalized breast epithelial. Methylation of H3K4 histones at seven regions (A1–A7) of the *SOD2* promoter and at *GAPDH* in breast cell lines was determined using chromatin immunoprecipitation (ChIP). Fold enrichments of dimethyl H3K4 at these regions were calculated as described under Methods and materials. Values given are the averages of three independent experiments with the standard error. (* $P<0.05$ between MCF-10A and breast cancer cell lines at respective amplicons. For individual statistical analysis between MCF-10A and breast cancer cell lines see Supplemental Table S1.)