Elevated *Sod2* Activity Augments Matrix Metalloproteinase Expression: Evidence for the Involvement of Endogenous Hydrogen Peroxide in Regulating Metastasis[1]

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ABSTRACT

Purpose: **Elevated manganese superoxide dismutase (*Sod2*) levels have been reported to be associated with an increased frequency of tumor invasion and metastasis in certain cancers, and the aim of this study is to examine the molecular mechanisms by which this occurs.**

Experimental Design: Sod2 **and catalase overexpressing HT-1080 fibrosarcoma cell lines were used to evaluate the H_2O_2-dependent regulation of matrix metalloproteinase (MMP)-1 promoter activity, mitogen-activated protein (MAP) kinase signaling, DNA-binding activity, and MMP mRNA levels. The invasive and metastatic potential of *Sod*2 overexpressing cells was characterized using subrenal capsular implantation or tail vein injection of tumor cells into nude mice, respectively.**

Results: **Our data reveal that *Sod2* overexpression increases the DNA-binding activity of transcription factors critical for MMP expression but also enhances MMP-1 promoter activity via the Ras//MAP/extracellular signal-regulated kinase (MEK) signaling cascade. A single nucleotide polymorphism that creates an Ets site at position −1607 bp confers *Sod2*-dependent MMP-1 promoter activity. *Sod2* overexpression also increases the mRNA levels of MMPs-2, -3, -7, -10, -9, -11 and enhances the metastatic potential of fibrosarcoma cells when implanted in immunodeficient mice. The *Sod2*-dependent increases in AP-1 and SP-1 DNA-binding activity, MMP-1 promoter activity, general MMP expression, and collagen degradation can be reversed by the hydrogen peroxide-detoxifying enzyme, catalase.**

Conclusion: **MMPs play a critical role in the process of stromal invasion and metastasis, and these findings suggest that the association between increased *Sod2* and poor prognosis in certain cancers may be attributed to elevated MMP production.**

INTRODUCTION

Studies from this and other laboratories have shown that overexpression of *Sod2* inhibits cellular proliferation both *in vitro* and *in vivo* (1). These findings and the apparent decrease in *Sod2* levels in numerous tumor cell lines as compared with their corresponding nonmalignant counterpart have led to the proposal that *Sod2* may be a tumor suppressor. However, a number of recent reports have demonstrated that elevated levels of *Sod2* are found in thyroid tumors (2), central nervous system tumors (3), and acute leukemias (4) and are also correlated with an increased frequency of invasion and metastasis of gastric and colorectal carcinomas (5, 6). These disparate findings have led us to investigate the mechanistic rationale for the increased metastatic capacity of tumor cells overexpressing *Sod2*. *Sod2* is localized to the mitochondria and is one of three mammalian Sods that functions to dismute superoxide ($O_2^{\cdot-}$) to H_2O_2 at near diffusion-limiting rates. It has been established that *Sod2*-dependent H_2O_2 production contributes to the signaling mechanism that regulates the expression of MMP[3]-1 (7, 8). MMPs are major contributors to stromal degradation involved in tumor invasion (9). We, therefore, tested the hypothesis that *Sod2* overexpression enhances the metastatic capacity of tumor cells *in vivo* by modulating the expression of MMP-1 and potentially other MMP family members. The results presented here indicate that the increased metastatic potential of *Sod2*-overexpressing tumor cell lines may be attributed to their enhanced MMP production.

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[3] The abbreviations used are: MMP, matrix metalloproteinase; SOD, superoxide dismutase; *Sod2*, manganese SOD; CMV, cytomegalovirus; MAP, mitogen-activated protein; MAPK, MAP kinase; hMMP, human MMP; RT-PCR, reverse transcription-PCR; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor; SRC, subrenal capsular; MEK, MAP/extracellular signal-regulated kinase; GAPDH, glyceraldehyde phosphate dehydrogenase.

MATERIALS AND METHODS

Cell Culture and Reagents. Human fibrosarcoma HT-1080 cells were cultured in MEM supplemented with 10% FCS, 1000 units/ml penicillin, 500 μg/ml streptomycin, and 1 mg/ml neomycin, in a 37°C humidified incubator containing 5% CO_2. HT-1080 fibrosarcoma cell lines transfected with CMV (empty vector) *Sod2* and/or catalase were described previously in detail (10). Cells were treated with recombinant human TNF (R&D systems, Minneapolis, MN). Gelatin zymography, SOD activity and RT-PCR analysis were performed as described previously (8, 10).

Analysis of Metastatic Tumor Growth in Nude Mice. For the determination of metastatic potential, 1×10^6 cells in a 0.2-ml volume of 1X PBS were injected into the tail veins of male athymic mice at 3–4 weeks of age. Mice were sacrificed 21 days later by the injection of a lethal dose of Nembutal, followed by cervical dislocation. Lungs were stained by injecting fekete's solution intratracheally, or they were formalin fixed and paraffin embedded. We used Masson's trichrome stain to detect collagen deposition as described previously (11).

SRC Growth of Tumors. In brief, cell lines were grown to confluence and harvested. Cells were converted to solid tumor form by centrifugation into a pellet and exposure of the cell pellet to 15 μl of fibrinogen (50 mg/ml) and 10 μl of thrombin (50 u/ml) for 30 min at 37°C. Fibrin clots were then cut into pieces ~1.5 mm in diameter and each piece implanted under the kidney capsule of a severe combined immunodeficient (SCID) mouse. Tumor size was measured using a dissecting microscope equipped with an ocular micrometer during survival laparotomies on days 10 and 20 after tumor implantation. A minimum of four replicate mice per group were analyzed.

MMP Analysis. RT-PCR analysis were performed as described previously (8). The following primer sets were used and the optimal annealing temperature and expected size of the PCR product are indicated: MMP-1, 55°C, 550 bp, 5′-GGA GGA AAT CTT GCT CAT, 3′-CTC AGA AAG AGC AGC ATC; MMP-2, 57°C, 1389 bp, 5′-CTA CGA TGA TGA CCG CAA GTG, 3′-AAA ACA AGA CCC AAA GAA AAA; MMP-3, 55.6°C, 403 bp, 5′-CCC ACT CTA TCA CTC ACT CAC, 3′-AGC TCG TAC CTC ATT TCC TC; MMP-7, 55°C, 420 bp, 5′-TCT TTG GCC TAC CTA TAA CTG G, 3′-CTA GAC TGC TAC CAT CCG TC; MMP-9, 60°C, 409 bp, 5′-GCT TCA TCC CCC TCC CTC CCT TT, 3′-TGA GAA CCA ATC TCA CCG ACA GGC; MMP-10, 54.4°C, 750 bp, 5′-CCC ACT CTA CAA CTC ATT CAC, 3′-CCA TAT CTG TCT TCC CCC TAT C; MMP-11,55°C, 1031 bp, 5′-GAA GAC GGA CCT CAC CTA CA, 3′-CAG AGC CTT CAC CTT CAC AG; MMP-12, 55°C, 261 bp, 5′-TCA CGA GAT TGG CCA TTC CTT, 3′-TCT GGC TTC AAT TTC ATA AGC; and MMP-13, 53.1°C, 684 bp, 5′-CCA ACC CTA AAC ATC CAA AAA C, 3′-TAG CTC TTC TTC CCC TAC CC.

MMP-1 Promoter Deletion Constructs. The hMMP-1 promoter/luciferase reporter plasmids full-length, −3830, −3292, −2942, −2002, −1193, −1546, and −517 used in this study contained the *firefly luciferase* gene under the transcriptional control of the hMMP-1 promoter and were provided by Dr. Constance Brinckerhoff. Additional 2G deletion constructs (−1902, −1802, −1702, −1602) were prepared by a PCR method using MMP-1 promoter-specific oligonucleotide primers introducing a *Xho*I site at the 5′ end of the desired deletion product and a *Hin*dIII site at the 3′ end of the deletion PCR product. The −2002 hMMP-1 promoter/luciferase plasmid (pGL3basic) was the template for the PCR. The resulting PCR products were enzyme-digested with *Xho*I and *Hin*dIII and subsequently subcloned into the *Xho*I/*Hin*dIII sites of the pGL3basic reporter vector (Promega, Madison, WI).

Transient Transfections of hMMP-1 Promoter Constructs and Dominant Negative Constructs. Cell lines were transfected with the various pGL3-MMP-1 (1G) or pGL3-MMP-1 (2G) constructs (8) and pCMV.SPORTβ-gal using LipofectAMINE Plus reagent according to manufacturer's instructions. Details of the use and construction of the pAdTrack-RasN17 has been described previously (12). The dominant/negative (S217A) MEK-1 cDNA was kindly provided by Dr. M. J. Weber of the University of Virginia, Health Sciences Center and has been previously described (13). The S217A MEK-1 cDNA was then excised from pBABE-PURO by digestion with *Bam*HI and *Eco*RI and inserted into a previously digested DIVA-CMV. The cells were lysed 18 h posttransfection, and the luciferase reporter activity was determined using the Promega assay system. All of the results were reported after normalization for transfection efficiency by measuring β-galactosidase activity.

Statistics. ANOVA with $\alpha = 0.05$ was used for processing the data. A two-sample *t* test was used as posttest unless otherwise indicated.

RESULTS

Identification of Redox-responsive MMP-1 Promoter Region. We have previously reported that the *Sod2*-dependent production of H_2O_2 enhances the activity of MMP-1 promoter containing the 2G SNP that creates a guanine base (G) insertion producing an Ets-binding site and that, consequently, increases MMP-1 transcriptional activity (8). Deletional analysis of the full-length promoter containing the 2G polymorphism indicates that a region from −2002 to −1546 is required for optimal basal and *Sod2*-dependent MMP-1 promoter activity (Fig. 1*A*). This region contains the polymorphic Ets consensus motif and has been shown to be largely responsible for the increased activity of the polymorphic MMP-1 promoter sequence. Deletion of the region from −2002 to −1546 in the 1G promoter also decreases its activity in response to *Sod2* overexpression. Therefore, the Ets-binding site at position −1607 and other elements in the region from −2002 to −1546 play an important role in regulating the redox-responsiveness of the MMP-1 promoter. To further define the regulatory elements responsible for the redox sensitivity of the MMP-1 promoter, 100-bp deletions were made in the region from position −2002 to −1546 in both the 1G- and 2G-containing promoters (Figs. 1, *B* and *C*). As in Fig. 1*A*, there is a dramatic increase in the promoter activity when *Sod2* is overexpressed. However, the sequential deletions affected both the basal and redox-responsive MMP-1 promoter activity in terms of fold loss of expression to a similar level with one exception (Fig. 1*D*). The most dramatic and statistically significant loss in redox-responsiveness (25-fold) was observed on the deletion of the region from −1702 to −1602 in the 2G construct

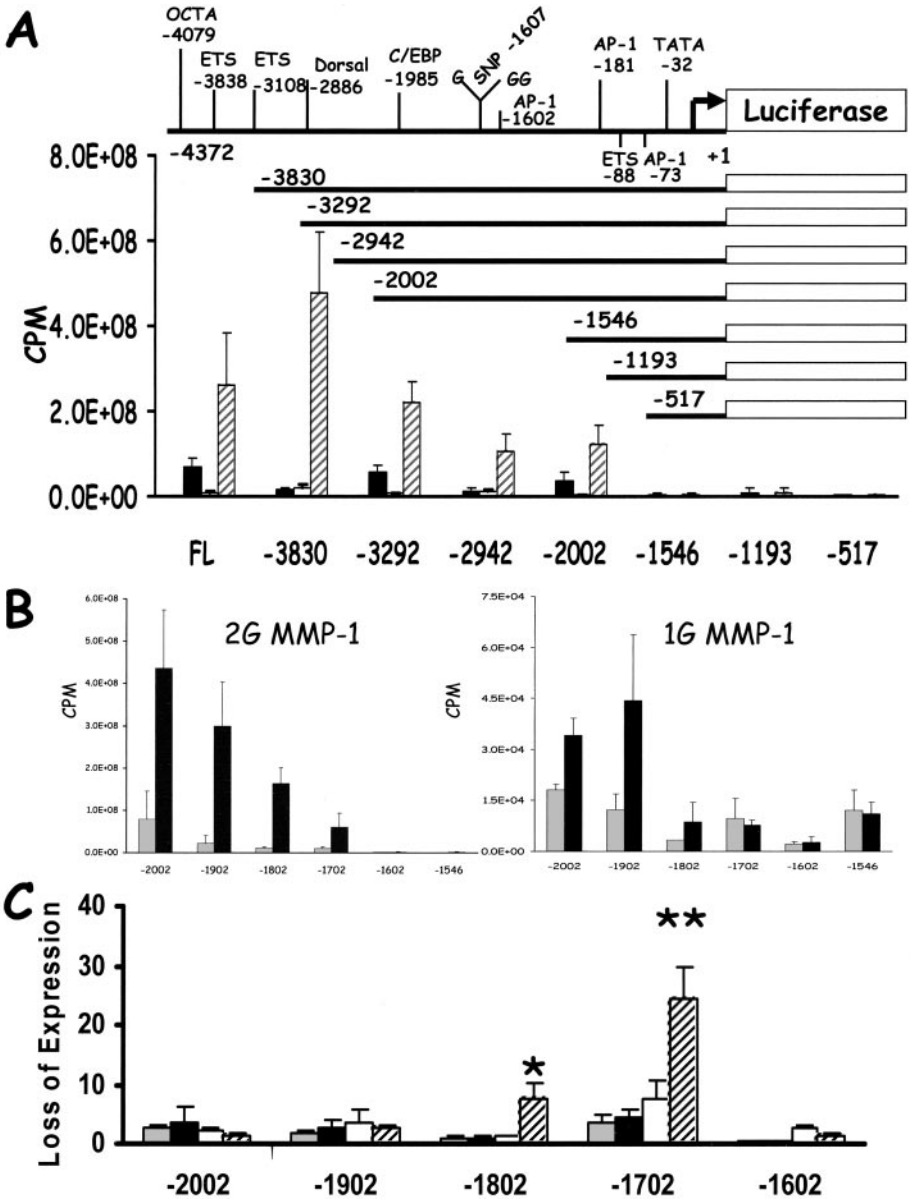

Fig. 1 The identification of *Sod2*-responsive region of the hMMP-1 promoter and signaling molecules. *A*, *top*, a schematic representation of promoter constructs tested and the location of known transcription factor consensus sequences and polymorphisms. *Bottom*, control (CMV) or *Sod2*-overexpressing (HT-15) cells were cotransfected with the various pGL3-MMP-1 2G or 1G constructs and pCMVβ-GAL and assayed for luciferase activity. CMV 1G (*gray*, not visible), HT15 1G (*black*), CMV 2G (*white*), HT15 2G (*hatched*). *B*, MMP-1 promoter deletion analysis of regions from −2002 to −1546. *Left*, cells were transfected with the indicated MMP-2G constructs and treated as described above. *Right*, similar to left panel, only with MMP-1G constructs. CMV (*gray*) and HT15 (*black*). C, identification of the *Sod2*-responsive region of the MMP-1 promoter. Data from *B* are expressed in terms of loss of fold expression when the indicated region is deleted and legend is as in *A*, *bottom panel*. ∗, $P < 0.05$; ∗∗, $P < 0.01$ when compared with CMV-transfected cell lines. All of the data are expressed as cpm and have been normalized for β-galactosidase activity unless otherwise indicated. Values are the mean ± SE of four independent experiments.

that was not observed in the 1G construct. This finding indicates that the redox-responsive element encompasses the 2G polymorphism containing the Ets consensus-binding motif.

Sod2-dependent Regulation of Ras/MAPK/AP-1. We previously demonstrated the importance of ERK1,2 in the MnSOD-dependent regulation of MMP-1 (8). By the use of dominant negative inhibitor constructs, we report that both Ras and MEK-1 also play a role in the MnSOD-mediated regulation of MMP-1. The dominant negative N17Ras decreased MMP-1 promoter activity in both the CMV and HT15 cells relative to a control vector protein (Fig. 2*A*, *left panel*). Cotransfection of the pGL3-MMP-1 2G reporter plasmid with the dominant negative MEK-1 construct significantly blocked MMP-1 2G promoter activity both in CMV and HT15 cells (Fig. 2*A*, *right panel*).

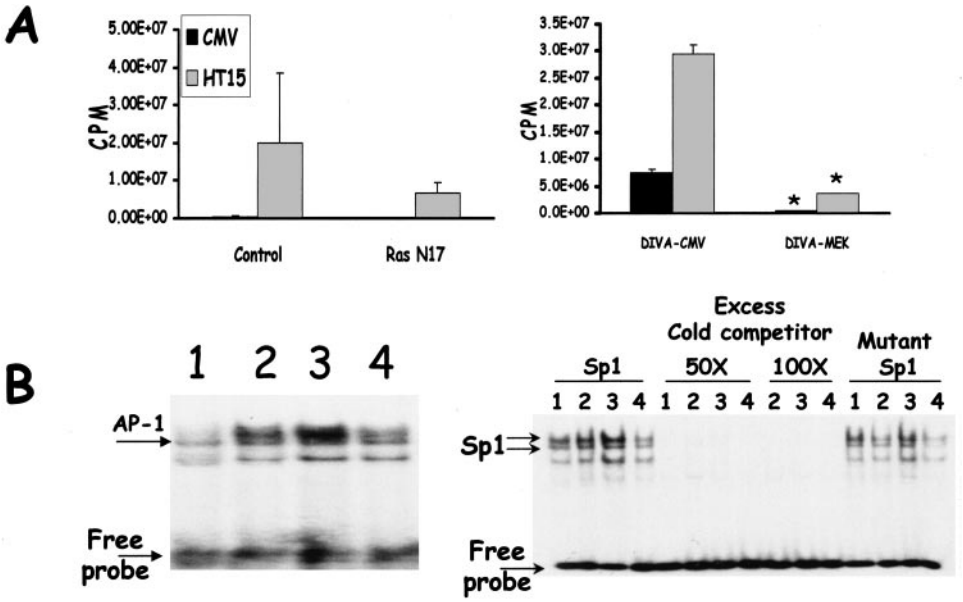

Fig. 2 *A*, the effect of dominant negative Ras/MEK constructs on the *Sod2*-dependent increases in MMP-1 promoter activity. *Left panel*, control (CMV) and MnSOD-overexpressing (HT15) were transfected with pGL3-MMP-1 2G promoter or transfected with pGL3-MMP-1 2G and a dominant negative mutant of Ras, pAdTrack-RasN17. Results are shown as cpm. *Right panel*, CMV or HT15 cells were transfected with pGL3-MMP-1 2G construct and either control vector (DIVA-CMV) or a dominant negative mutant of MEK-1 (DIVA-MEK1). Results are express as the mean of four independent experiments normalized to β-galactosidase activity and protein content ± SE. ∗, $P < 0.001$ when compared with cell lines transfected with the DIVA-CMV. *B*, *Sod2* modulates AP-1 and SP-1 DNA-binding activity and MMP expression. *Sod2*-regulation of Transcription factors extracted from HeLaTat (*Lane 1*), CMV (*Lane 2*), HT15 (*Lane 3*), and HT15mCAT (*Lane 4*) cell lines. *Left panel*, electrophoretic mobility shift analysis of the transcription factor AP-1 in the above mentioned cell lines. *Right panel*, DNA-binding activity of the transcription factor Sp1 was analyzed by incubating with a ^{32}P-labeled Sp1 oligonucleotide in either the presence or the absence of excess cold competitor (50-fold or 100-fold), or excess of cold mutant oligonucleotide. All of the results were visualized by exposure to Kodak BioMax-MS film.

Thus, these studies indicate that MnSOD-dependent regulation of MMP-1 signals though the Ras/MEK/ERK pathway.

The Ras/MAPK pathway has been shown to mediate the activation of c-*fos* gene expression, which, in turn, forms the AP-1 transcription factor with Jun family members. AP-1 DNA binding activity increased when *Sod2* was overexpressed and was reversed by coexpression of mitochondrial catalase (Fig. 2*B*, *left panel*). The proximal AP-1 element located near position −72 plays a major role in the transcriptional regulation of the *hMMP-1* gene and is found near this position in each of the inducible hMMP promoters (14). However, deletion analysis of the full-length promoter suggests that elements far upstream of the −72 AP-1 impact the activity of the MMP-1 promoter to a greater degree. Furthermore, an AP-1 consensus motif is also found at position −1602 adjacent to the Ets SNP site. Although not found in the MMP-1 promoter, SP-1 elements have also been shown to be critical to the regulation of other MMP family members (14), and analysis of SP-1 DNA-binding activity also exhibited sensitivity to the *Sod2*-dependent production of H_2O_2 (Fig. 2*B*, *right panel*).

Redox-regulation of Inducible MMP Family Members. MMP-1 expression has been shown to be sensitive to the modulation of intracellular H_2O_2 (7–9). The promoter regions of inducible *MMP* genes have many conserved regulatory elements (14) and may also respond to changes in the steady-state concentrations of hydrogen peroxide similar to MMP-1. To test this hypothesis, we evaluated the expression of the inducible MMPs using RT-PCR in cell lines that overexpress *Sod2* and catalase (mitochondrial or cytosolic) or a combination of the two (15). The mRNA levels of MMP-2, -3, -7, -9, -10, -11, -12, and -13 were increased in the *Sod2* overexpressors when compared with control cell lines (Fig. 3*A*). Furthermore, the *Sod2*-dependent increases in MMP expression were attenuated by the coexpression of catalase in either the mitochondrial or the cytosolic compartment. Catalase alone also decreased the basal expression of several of the MMP family members including MMP-2, -3, -7, -10, -12. These data indicate that many of the MMP family members respond similarly to *Sod2*-dependent production of H_2O_2 and that *Sod2* may regulate a broad spectrum of MMPs and function as a "global" redox regulator of metalloproteinases.

Loss of MMP Expression by Down-Regulation of Sod2. To establish that *Sod2* can modulate MMP-1 expression in other tumor cell lines, we analyzed MMP-1 levels in HeLa Tat cell lines that show a 50% decrease in basal *Sod2* activity. The HIV Tat protein has been shown to specifically interfere with *Sod2* transcription and decrease *Sod2* expression (16). Both basal and TNF-induced *Sod2* activity were decreased in the HeLa Tat-expressing cell lines when compared with the HeLa parental cell line (Fig. 3*B*). The decrease in *Sod2* activity was also sufficient to prevent the TNF-mediated induction of both MMP-1 and MMP-3 mRNA expression. To determine whether the reduction in MMP expression was attributable to the Tat-mediated decrease in *Sod2* expression HeLa Tat-expressing cells were trans-

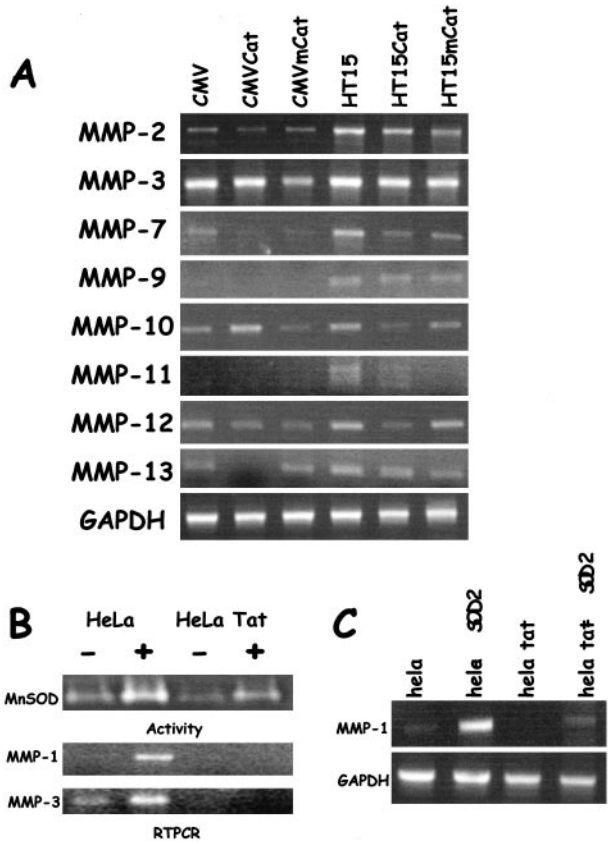

Fig. 3 Redox-dependent expression of inducible MMPs. *A*, RT-PCR analysis of MMP levels from CMV, CMVCAT, CMVmCAT, HT15, HT15CAT, and HT15mCAT. See "Materials and Methods" for details. *B*, analysis of *Sod2*, MMP-1, and MMP-3 from HeLa and HeLa Tat cells. *Top panel*, nondenaturing Sod PAGE of HeLa and HeLa Tat cell extracts before and after overnight treatment with TNF (10 ng/ml) treatment. Ten μg of total cell protein were loaded per lane. *Lower panel*, RT-PCR analysis of MMP-1 and MMP-3 treated as described above. *C*, Sod2 overexpression reverses the Tat-dependent inhibition of MMP-1 expression in HeLa cells. RT-PCR analysis of MMP-1 and GAPDH HeLa and HeLa Tat transfected with or without *Sod2*.

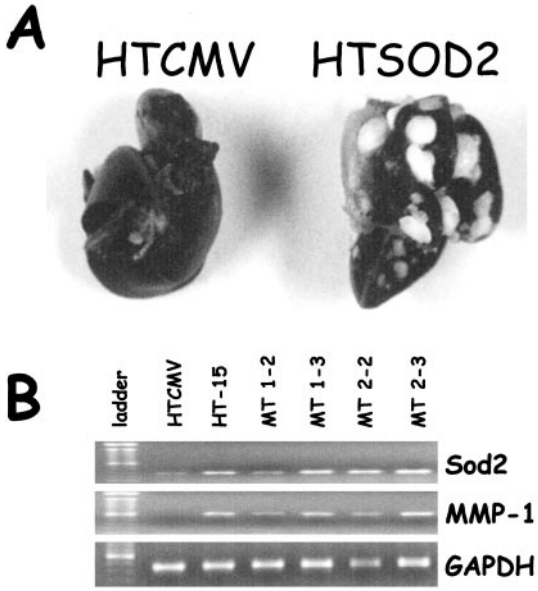

Fig. 4 *In vivo* analysis of *Sod2*-overexpressing tumor cell lines. *A*, *Sod2* confers metastatic potential on fibrosarcoma cells. Appearance of lung metastases from mice that were given i.v. injections with a heterogeneous population of *Sod2*-overexpressing or HTCMV (control) cell lines. Lungs were perfused via the trachea with a 15% solution of india ink and fixed in fekete's solution. *B*, RT-PCR analysis of MnSOD, MMP-1, MMP-3, and GAPDH from HTCMV and HT*Sod2*-isolated metastatic nodules as shown in *A*. *MT 1–2*, mouse 1 nodule 2; *MT 1–3*, mouse 1 nodule 3; *MT 2–2*, mouse 2 nodule 2; *MT 2–3*, mouse 1 nodule 3.

fected with a *Sod2* expression vector and were shown to partially recover MMP-1 expression (Fig. 3*C*). *Sod2* overexpression in HeLa cells was also shown to increase the basal expression of MMP-1. These studies further support our previous findings that *Sod2* is involved in the signaling pathways that lead to the expression of MMPs.

Redox-dependent Regulation of Invasion and Metastasis. We have previously established that *Sod2*-dependent production of H_2O_2 can enhance MMP production (8). A consequence of elevated MMP production in response to *Sod2* overexpression would be an increase in both the invasive and metastatic potential of tumor cells. To test this hypothesis previously established control and *Sod2*-overexpressing HT-1080 fibrosarcoma cell lines were injected into the tail-vein of NCR nude mice and lungs were inspected for metastases 30 days after injection. *Sod2*-overexpressing HT-1080 cell lines showed a dramatic increase in their ability to colonize the lung when compared with the control HTCMV (empty vector) cell line (Fig. 4*A*). Lung metastases were observed in 13 of the 18 mice that were given injections of the heterogeneous population of *Sod2*-overexpressing cell lines as compared with only 2 of the 18 in the control group (Table 1). Analysis of several metastatic nodules by RT-PCR showed increases in *Sod2* (3 of 4) and MMP-1 (4 of 4) relative to the control CMV cell line (Fig. 4b). H&E staining did not show any phenotypic differences between control and Sod2-overexpressing metastatic foci besides size and number of metastatic nodules present in the lung sections (data not shown). The data for three independent experiments are summarized in Table 1 and clearly demonstrate that the metastatic potential of HT-1080 fibrosarcoma cell lines is dramatically enhanced in response to *Sod2* overexpression.

SRC xenotransplantation was used to monitor the potential of the various redox-engineered cell lines to degrade collagen (the target of many matrix-degrading metalloproteinases). SRC xenotransplantation of the control and *Sod2*-overexpressing tumor cells was performed in ICR severe combined immunodeficient mice. No differences were observed in the rate of tumor formation between the various cell lines. To determine whether modulation of Sod2 levels affect collagen deposition, sections were stained with Masson's trichrome. Representative micrographs of stained SRC xenotransplantation from tissue are shown in Fig. 5. *Sod2*-overexpressing tumor cells (HT15 = 15-fold increase in *Sod2* activity) showed a prominent decrease in trichrome staining relative to control (HTCMV) cell line (Fig. 5). We have demonstrated that the H_2O_2-detoxifying enzyme, catalase, attenuates the *Sod2*-dependent increases in MMP ex-

Table 1 Metastatic potential of *Sod2*-overexpressing and HT-1080 cell lines

Cells	Incidence (no. of mice with lung metastasis/no. of mice given injections)				No. of lung metastatic nodules[a]																	
	Exp[b] 1[c]	Exp 2[d]	Exp 3[e]	Total	Experiment 1							Experiment 2						Experiment 3				
HT-CMV	1 of 6	0 of 6	1 of 6	2 of 18	5	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
HT-SOD2	5 of 6	3 of 6	5 of 6	13 of 18[e]	>100	8	0	>100	>100	NA	45	75	0	0	0	NA	81	37	0	0	21	66

[a] Each value represents the number of colonies per mouse lung.
[b] Exp, experiment; NA, lungs had metastatic nodes but were not available for quantification.
[c] Lungs were perfused with india ink and metastatic nodes counted macroscopically.
[d] Lungs were paraffin-embedded, stained with hematoxylin and eosin and examined microscopically.
[e] $P < 0.00001$ χ^2 analysis was used to test the difference between metastatic potential of HTCMV and HT-Sod2 cell lines.

pression. Coexpression of catalase in the *Sod2*-overexpressing tumor cell lines reversed the decrease in collagen deposition at the tumor-kidney interface. Of the 12 mice evaluated (4/tumor type) all of the *Sod2*-overexpressing tumors demonstrated a clear loss of collagen deposition at one or more areas of the tumor-kidney interface (Fig. 5; see ×10 and ×20 sections). These *in vivo* studies indicate that *Sod2*-dependent production of H_2O_2 can modulate both the metastatic potential and the degradation of collagen by fibrosarcoma cells.

DISCUSSION

The *Sod2*-dependent production of H_2O_2 contributes to the signaling pathway that modulates not only MMP-1 expression but also many of the inducible MMP family members. We have also identified a region within the MMP-1 promoter between position −2002 and −1546 that is critical for optimal basal and redox activation of MMP-1 promoter activity. Furthermore, the *Sod2*-sensitive signaling cascade uses the Ras/MAPK/AP-1 pathway and leads to an increase in the metastatic capacity and invasive potential of HT-1080 fibrosarcoma cells that can be attenuated by the H_2O_2-detoxifying enzyme, catalase.

The Ets and AP-1 motif at position −72 and −88, respectively, have been shown to contribute to the induction of MMP-1 promoter (17). However, in the present study, the Ets and AP-1 promoter elements downstream of −1546 minimally contribute to MMP-1 promoter activity. The SNP at position −1607 has been shown to greatly enhance MMP-1 promoter activity (18). Deletion of the region between −2002 and −1546 containing the Ets binding site at position −1607 decreases *Sod2*-dependent promoter activity nearly 30-fold. An equivalent fold loss in promoter activity is observed both in the control and *Sod2*-overexpressing cell lines until the region between −1702 to −1602 is deleted (Fig. 1*C*). This deletion results in a 25-fold loss in promoter activity in the *Sod2*-overexpressing cell lines. A similar deletion in the 1G construct shows no difference in fold loss of activity between the *Sod2* and control cell lines (Fig. 1, *B* and *C*). Although there appears to be a loss in *Sod2*-dependent promoter activity in the 1G construct when the region from −1902 to −1802 is deleted, this difference was not found to be statistically significant. Furthermore, other elements in the full-length promoter contribute to its maximal activity, but none show such a profound loss in activity as compared with the deletion of the Ets-binding motif. Thus, the 2G SNP that creates an Ets consensus-binding motif is the essential element that confers the maximum redox-responsiveness to the MMP-1 promoter. It has been suggested that Ets may act as a sensor for mitochondrial function by its ability to regulate the transcriptional activity of the mitochondrial ATP synthase (19). Thus, it is possible that *Sod2* may alter mitochondrial function and thereby modulate Ets activity.

The DNA-binding activity of both Ets and AP-1 is redox sensitive (20) and responsive to MAPK signaling (14). The dominant negative isoforms of Ras and MEK-1 attenuate the *Sod2*-dependent increase in MMP-1 expression (Fig. 1, *B* and *C*), suggesting that the Ras/MAPK/AP-1 signaling cascade is quite sensitive to *Sod2*-dependent H_2O_2 production. We have reported that the *Sod2*-dependent induction of MMP-1 signals through the activation of ERK1/2 (8). The limited inhibitory effect of N17Ras relative to the dominant-negative MEK-1 (Fig. 2*B*) may be explained by the presence of Ras-independent MEK/ERK activation cascade similar to that reported for nerve growth factor activation of ERK (21).

Antioxidants such as the glutathione precursor *N*-acetyl cysteine block MMP production and metastasis (22–24), whereas selenium, an essential component of the H_2O_2-detoxifying enzyme glutathione peroxidase, inhibits invasion of HT1080 human fibrosarcoma cells and decreases MMP expression (25). Nishikawa *et al.* (26) have recently shown that the both i.v. and s.c. injection of catalase derivatives can inhibit the formation of experimental pulmonary metastases in mice. In addition, decreases in catalase activity have been correlated with the emergence of the malignant phenotype in mouse keratinocytes treated with the carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (27). The ability of catalase to attenuate both the basal and the *Sod2*-dependent MMP expression and collagen deposition further supports the importance of oxidants in regulating these processes.

The *Sod2*-dependent up-regulation of MMPs and their critical role in invasion and metastasis may explain why tumors with elevated levels of *Sod2* are more invasive (7, 8). Janssen *et al.* (28) evaluated adenocarcinomas from the stomach of 81 patients and showed a significant increase ($P > 0.007$) in *Sod2* levels relative to normal tissues. Malafa *et al.* (6) have shown that *Sod2* expression is increased in 93% of metastatic as compared with 44% of nonmetastatic gastric tumors. The present observation that *Sod2* overexpression mediates increased expression of numerous MMP family members suggests that *Sod2* modulates invasion and metastasis via multiple proteinases.

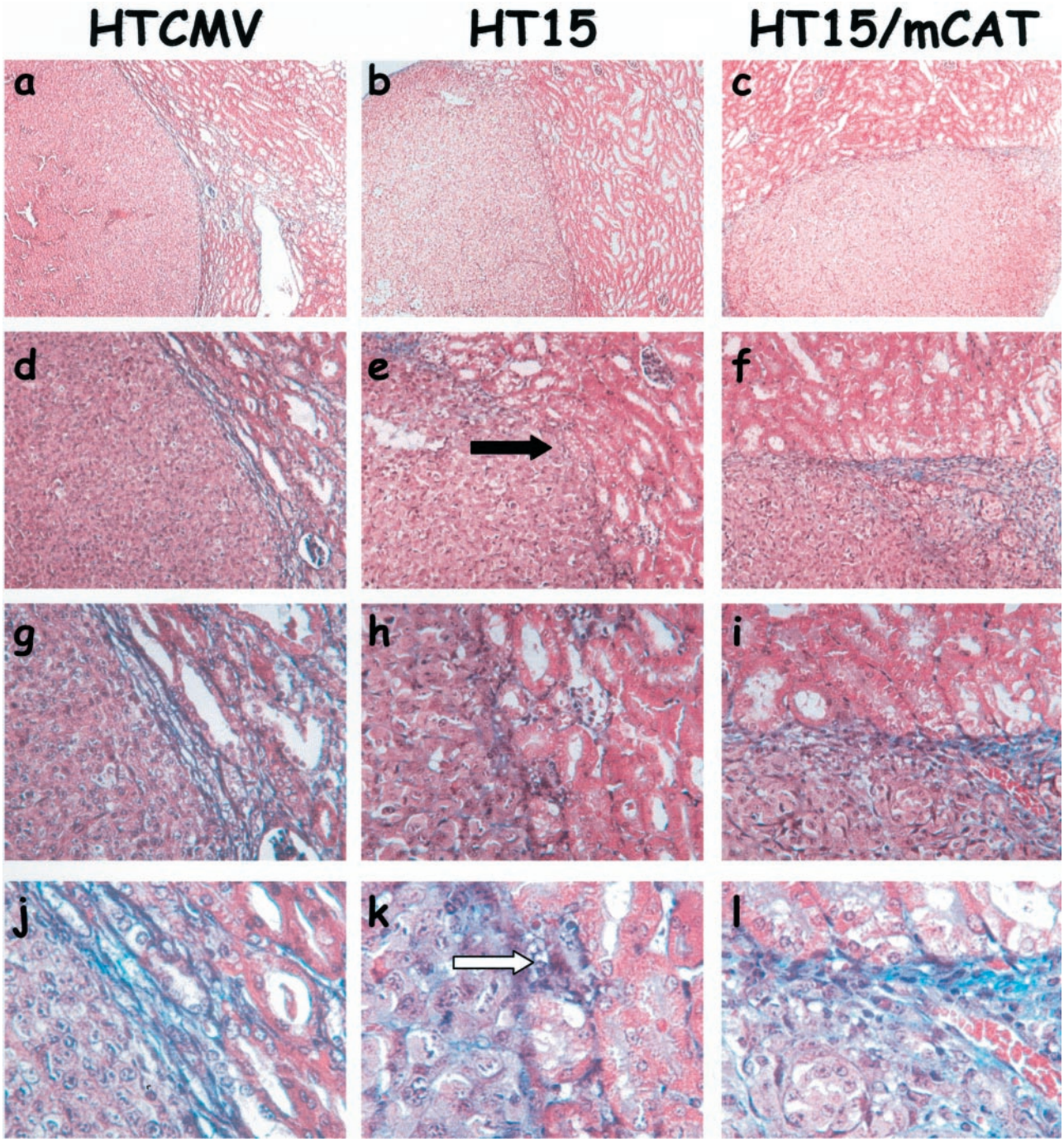

Fig. 5 Masson's trichrome staining of SRC of control and *Sod2*-overexpressing cell lines after 2 weeks. *a*, *d*, *g*, and *j*, HTCMV (control); *b*, *e*, *h*, and *k*, HT15 (15-fold increase in *Sod2*); *c*, *f*, *i*, and *l*, HT15/mCAt (*Sod2* and catalase coexpressors). *Light blue staining*, areas of collagen deposition. *Black arrow*, area showing regions in which there is a complete loss of collagen. *White arrow*, regions in which tumor cells are infiltrating the underlying renal parenchyma. *From top to bottom*, ×4, ×10, ×20, and ×40, respectively. Images are representative of four independent SRC xenotransplantations for each cell type.

These findings can explain the reports by several independent groups that *Sod2* levels may be used as prognostic parameters to evaluate the clinical outcome of patients with esophageal (28), colorectal (5, 29), and gastric cancers (5, 6, 28).

Sod2 is essential for MMP-13 (the murine functional homologue of MMP-1) expression in mouse embryonic fibroblasts (8) and also dramatically enhances the transcription of a MMP-1 promoter containing a SNP that has been linked to increased

incidence of metastases (30). A *Sod2* SNP has also been linked to an increase in breast cancer risk in premenopausal women with a low consumption of dietary sources of antioxidants (31). The *Sod2* polymorphism GTT→GCT leads to the insertion of either a Val or Ala in the mitochondrial leader sequence, respectively, and it has been proposed that the presence of the *Ala* allele may enhance the rate of mitochondrial import as well as its activity. Thus, we have identified two proteins that interact functionally and have been linked to increased cancer incidence and metastasis. Future studies will be directed at evaluating whether individuals carrying both the *Sod2* and the MMP-1 polymorphism may be at risk for developing metastatic cancers.

The present study is in contrast to reports from a number of laboratories, including our own, that have demonstrated the antitumoral properties of *Sod2* overexpression (1). Thus, it appears that, on the one hand, *Sod2* overexpression inhibits tumor cell growth but, on the other, promotes metastasis. Oberley *et al.* (32) have recently reported that *Sod2* levels are elevated at the invasive edge of primary prostate cancer. *Sod2* overexpression has also been shown to suppress metastasis of a mouse fibrosarcoma cell lines (33). The difference in the response of a specific cell type to *Sod2* overexpression may be dependent on the ability of the cell to detoxify hydrogen peroxide. Li *et al.* have shown that stable transfection of *Sod2* can result in compensatory increases in either catalase or glutathione peroxidase (34); thus, the steady-state concentration of hydrogen peroxide would remain relatively constant. We have previously established that both catalase and glutathione peroxidase activities are unchanged and that the steady-state concentration of hydrogen peroxide is increased (8) in the *Sod2*-overexpressing cell lines used in the present study (15). Ho *et al.* have recently demonstrated that *Sod2* levels are elevated and that catalase decreased in primary lung tumors when compared with adjacent normal tissue; elevated *Sod2* levels and decreased catalase, together, may lead to increased hydrogen peroxide production (35). Thus, differences in a tumor cell's response to *Sod2* expression likely reflect the cell's ability to detoxify hydrogen peroxide and, in turn, will dictate its tumorigenic and metastatic potential.

Sod2 is generally thought to be low in primary tumors, as has been clearly demonstrated both *in vivo* and *in vitro*. Bostwick *et al.* (36) have shown that *Sod1, Sod2*, and catalase have lower expression in prostatic intraepithelial neoplasia and prostate carcinoma relative to benign epithelium, which supports the notion that decreased antioxidant enzyme activity is associated with these lesions. However, many of these studies fail to assess the involvement of metastatic tissue with respect to their antioxidant enzyme status. An analysis of prostate-related metastatic lesions by Oberley *et al.* (32) showed a moderate-to-heavy labeling for *Sod2* and markers of reactive oxygen and nitrogen species with no significant alterations in immunoreactive *Sod1*, glutathione peroxidase, or catalase. These contradictory outcomes may also reflect the stage of tumor progression. It is reasonable to hypothesize that low levels of *Sod2* may initiate or promote the neoplastic process. Low levels of *Sod2* in tumor cells may not adequately detoxify superoxide, resulting in an enhancement of the mutagenic potential of the cell. Overexpression of *Sod2* during the promotion stage may prevent further mutagenesis and inhibit tumor cell growth. In contrast, the elevated *Sod2* levels observed in a variety of metastatic cancers may be a response of the tumor to inflammatory cytokines and growth factors produced as a result of the host antitumoral immune response.

The present study indicates that the *Sod2*-dependent production of H_2O_2 plays an important role in regulating MMP expression, tumor invasion and metastasis. The importance of *Sod2* in regulating MMP production may be even more relevant in the tumor/stromal microenvironment. *In vivo*, MMP production may be exacerbated by inflammatory cytokines that induce *Sod2* expression and are released at the periphery of the tumor. Furthermore, tumors with elevated levels of *Sod2* may be protected from free-radical-mediated tumor killing initiated by chemo- or radiation therapy. It is also possible that these therapies may promote tumor expansion by enhancing *Sod2*-dependent H_2O_2 production and subsequent MMP expression. These studies would suggest that assessment of *Sod2* and MMP status in cancer patients might identify individuals who may respond poorly to therapeutic strategies using redox-cycling drugs or ionizing radiation. Furthermore, efficient antioxidant-based therapeutic strategies may prove useful for the treatment of metastatic disease.

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