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Manganese superoxide dismutase (Sod2) and redox-control of signaling events that drive metastasis

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Abstract

Manganese superoxide dismutase (Sod2) has emerged as a key enzyme with a dual role in tumorigenic progression. Early studies were primarily directed at defining the tumor suppressive function of Sod2 based on its low level expression in many tumor types. It is now commonly held that loss of Sod2 expression is likely an early event in tumor progression allowing for further propagation of the tumorigenic phenotype resulting from steady state increases in free radical production. Increases in free radical load have also been linked to defects in mitochondrial function and metastatic disease progression. It was initially believed that Sod2 loss may propagate metastatic disease progression, in reality both epidemiologic and experimental evidence indicate that Sod2 levels increase in many tumor types as they progress from early stage non-invasive disease to late stage metastatic disease. Sod2 overexpression in many instances enhances the metastatic phenotype that is reversed by efficient H₂O₂ scavenging. This review evaluates the many sequelae associated with increases in Sod2 that impinge on the metastatic phenotype. The ability to use Sod2 to modulate the cellular redox-environment has allowed for the identification of redox-responsive signaling events that drive malignancy, such as invasion, migration and prolonged tumor cell survival. Further studies of these redox-driven events will help in the development of targeted therapeutic strategies to efficiently restrict redox-signaling essential for malignant progression.

INTRODUCTION

Sod2 expression during metastatic disease

While it has been known for some time that reactive oxygen species (ROS) are involved in tumor initiation and progression, the mechanisms and causes of enhanced ROS accumulation during tumorigenesis remain an area of active investigation. Tumor cells often display enhanced intracellular ROS levels and have adapted to cope and potentially utilize these changes in ROS for their normal function. In turn, it has been shown that many tumor cells display significant changes in antioxidant enzyme expression profiles. Whether this results as a consequence of changes in ROS levels or contributes to a shift in redox balance remains to be determined. Here we will focus on one important antioxidant enzyme of the mitochondria, manganese superoxide dismutase (Sod2) and the growing evidence that enhanced expression of Sod2 is associated with metastatic progression of some cancer types. Sod2 is the major mitochondrial enzyme responsible for dismutation of superoxide (O₂^{•-}), which primarily arises due to electron leakage from the mitochondrial electron transport

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chain. Following dismutation by Sod2, the resultant hydrogen peroxide (H_2O_2) is a more stable ROS compared to $O_2^{\cdot -}$ and can easily diffuse throughout the cell, where it may be further metabolized by catalase or peroxidases. The role of Sod2 and its changes in gene expression during tumorigenicity have been areas of intense investigation, yet due to its somewhat conflicting expression profile in different cancers, as well as its differential action on mechanisms of tumorigenesis, the role for Sod2 as either a tumor suppressor or oncogene is still being determined. For the most part it appears that Sod2 expression in some primary tumors is suppressed, whereas expression increases in more aggressive tumors and during metastatic progression.

In 1979 Oberley and Buettner first summarized the role of Sod2 expression in cancer cells and hypothesized a tumor suppressor role for Sod2. Early studies by the late Dr. Larry Oberley showed that Sod2 expression was decreased in cancer versus normal tissues and that this shifts the redox balance of tumor cells [1] to a more pro-oxidative state. Microarray studies of tumor specimen as well as cell lines confirm that Sod2 levels are significantly decreased in many of the cancers previously investigated by Oberley and others. For example, studies from prostate and leukemia specimen overwhelmingly show decreases in Sod2 expression compared to normal tissue controls (Table 1). For reviews on the loss of Sod2 during tumorigenesis and potential mechanisms, we refer the reader to the following articles [1-3].

With more data from subsequent studies on different tumor types, as well as tumors with varying stages of metastatic disease, the profile of Sod2 expression is increasingly becoming a more complicated picture and suggests a dichotomous role for this antioxidant enzyme in tumorigenesis. It appears that changes in Sod2 expression are dependent on cancer type, as well as progression and/or aggressiveness of the disease. Table 1 lists the number of publicly available microarray studies, collected by the online resource Oncomine.org, that show significant changes in Sod2 expression at the mRNA level compared to normal controls. Studies from kidney, brain & CNS, head & neck, colorectal, lymphoma, testicular seminoma & teratoma, gastric, skin squamous cell carcinoma and pancreatic cancers largely show increases in Sod2 expression when compared to normal tissues (Table 1). Independent studies have also shown increases in Sod2 expression in kidney cancer [4]; brain & CNS cancers [5-12]; head & neck, esophageal & oral squamous cell carcinomas [13-18]; mesothelioma & lung cancers [19-24]; gastric & colorectal cancers [13,25-31]; and pancreatic cancer [32]. In addition, many of these cancers display further increases in Sod2 expression during progression to metastatic disease. Examples of increases in Sod2 expression with increasing stage and grade of metastatic brain tumors from Oncomine.org microarray studies are shown in Figure 1. While data on patient outcome is not always readily available, it appears that in some cases enhanced Sod2 expression correlates with poor survival rate or recurrence. Independently published studies have shown increased Sod2 activity in advanced stages of head and neck squamous cell carcinoma with regional metastases [16], enhanced Sod2 expression in malignant mesothelioma [21]; and an association of increases in Sod2 expression with metastatic progression of pancreatic [32] gastric [25] colorectal [33] and oral squamous cell carcinoma [18]. In contrast, it appears that Sod2 levels further decrease with increasing metastatic stage of prostate cancer (Figure 1), while a recent studies suggest that increased Sod2 expression is associated with neuroendocrine differentiation of advanced stage prostate cancer [34] and increases in circulating Sod2 positively correlate with increased Gleason score of prostate cancer patients and tumor reoccurrence in the form of bone metastases [35].

To complicate the picture, some cancers display little or no significant changes in Sod2 expression between normal and primary tumor specimen (eg. lung, melanoma, myeloma, ovarian, cervical and esophageal cancers; Table 1) or conflicting changes in expression

dependent on study (sarcoma, liver and breast cancers; Table 1), yet data on some of these cancer types consistently show enhanced Sod2 expression that correlates with metastatic progression and poor outcome of disease. Studies from Oncomine.org include examples from metastatic lesions of breast, lung and bladder origin, where increased expression of Sod2 appears to correlate with increased stage and grade of these cancers (Figure 1, examples of metastatic breast shown). In metastatic bladder cancer Sod2 expression significantly correlates with stage (T stage, local invasion; and N stage, lymph node invasion) and grade of cancer [36] and we have shown that similar changes in Sod2 expression occur in a metastatic bladder cancer cell model [36]. Enhanced Sod2 expression appears to also correlate with metastatic progression in estrogen receptor (ER) negative breast cancers [37] and in malignant liver tumors when compared to benign tumor specimen [24].

Given these increases in Sod2 expression and potential correlation to outcome of the disease, researchers have suggested the use of Sod2 as a potential prognostic marker in a number of the above mentioned cancer types [12,14,17,18,22,26,28,33,38,39]. Further, some have suggested that changes in the circulating levels of Sod2 may also be useful as a diagnostic and predictive marker for certain cancer types [40,41].

It is now becoming clear that there is a dichotomous role for Sod2 during tumorigenesis. Sod2 may act as a tumor suppressor during the initial onset/proliferative stage of tumor initiation, yet once the tumor progresses to a more aggressive and invasive phenotype, Sod2 levels appear to positively correlate and contribute to enhanced metastatic behavior of cancer cells. Increased expression of Sod2 in metastatic cancer specimen, as well as in metastatic cell lines, appears to correlate with an increase in Sod2 activity, suggesting a functional role for this enzyme during metastatic progression [16,21,24,32,42]. However, many studies on patient samples do not include sufficient data on Sod2 activity, and it is therefore necessary to further establish that activity during metastatic progression positively correlates with enhanced Sod2 expression. For example, in Alzheimer's Disease it was shown that Sod expression was significantly increased in brains slices of afflicted patients, yet Sod activity was markedly reduced [43]. A recent manuscript by Shan *et al.* demonstrates that thioredoxin 1 (Trx1) expression is significantly enhanced in high grade prostate cancer specimen compared to low-grade samples, yet Trx1 activity is decreased in high grade tumors, due to enhanced oxidation and enzyme inactivation [44]. It has been reported that Sod2 transcription and post-transcriptional regulation account for a decrease in Sod2 activity following hyperoxic exposure in rat lungs[45]. A recent proteomic study identified two different Sod2 “spots” from medulloblastoma cell line protein extracts by 2-D electrophoresis, which were due to 2-oxo-histidine modifications of His-30 and His-31 on one of these Sod2 proteins[46]. It is not clear whether this modification impacts activity, however evidence from site-directed mutagenesis studies suggests that His-30 is an important amino acid involved in the hydrogen bond network in the catalytic domain of Sod2. A His-30-Asp substitution resulted in more efficient dismutation of $O_2^{\cdot-}$ by Sod2, due to inability of this mutant to be product-inhibited within the catalytic site[47,48]. Another known mechanism for inhibition of Sod2 activity is tyrosine nitration [49]. While evidence from the studies mentioned in the previous paragraphs appear to show that expression and enhanced Sod2 activity correlate in metastatic lesions, it remains to be investigated whether activity of Sod2 is altered in metastatic disease due to mechanisms such as nitration or oxidation.

With the advent of genome sequencing and identification of single nucleotide polymorphisms (SNP) a great deal of interest arose from the discovery of SNPs in the Sod2 gene. The Sod2 polymorphisms that emerged were soon followed by studies indicating their association with cancer risk. The primary SNP associated with cancer risk was Val(16)Ala

polymorphism in the mitochondrial signal peptide. Interestingly, the risk associated SNP (Ala) has been shown to be efficiently expressed and localized to mitochondria [50]. It has been suggested that increased epidemiologic cancer risk associated with the Val(16)Ala polymorphism may be attributed to its enhanced activity that alters the cellular redox environment to increase steady-state [H₂O₂] [51]. The Sod2-dependent shift in the cellular redox-state increases matrix turnover, inhibits cell death and drives redox-signaling. We refer the reader to a review by Bag and Bag [51] for a comprehensive discussion on this topic.

How do Sod2 levels change during metastasis?

Since much of the early research centered on the loss of Sod2 expression in a number of cancer types and its role as a tumor suppressor, more research has focused on the mechanisms that regulate its transcriptional down-regulation during tumorigenesis. Loss of Sod2 expression during tumorigenesis has been attributed to hypermethylation of its promoter [52,53], histone modification of the Sod2 gene in breast cancer [54] and potential chromosome instability of chromosome 6 during progression to cancer [2].

In this review, however, we explore the role of enhanced expression of Sod2 during metastatic progression and pro-metastatic signaling events (discussed below). A number of studies are starting to explore potential mechanisms for this dysregulation of Sod2 expression. We will only briefly touch on potential mechanisms that lead to enhanced Sod2 expression in aggressive/metastatic cancers, which include increases in ROS levels [55], changes in cytokine levels and transcription factor expression during tumorigenesis, that regulate the *Sod2* gene promoter. We refer the reader to the referenced articles for more detailed information.

An altered transcription factor profile in cancer cells may contribute to increases in Sod2. NFκB has been shown to directly act on and induce activity of the Sod2 promoter. Resistance to chemotherapy and radiation has been attributed to the activation of the NFκB pathway and subsequent induction of Sod2 transcription in a number of cancer cell lines [56,57]. Interestingly, a recent study by Dhar *et al.*, points to a bidirectional regulation of Sod2 by p53, which was shown to be dependent on NFκB and Sp1[58]. Low levels of p53 increase Sod2 transcription by enhancing NFκB binding to the *Sod2* promoter. Conversely, high p53 levels likely prevent binding of Sp1 to the *Sod2* promoter, inhibiting its constitutive activation. The authors conclude that high levels of p53 prevent Sod2 expression to enhance apoptosis, whereas low levels of p53 may enhance Sod2 levels to promote a pro-survival state. Similarly, it has been reported that p53 ^{-/-} fibroblasts have high Sod2 expression and activity, enhancing their resistance to oxidative stress[59]. This may explain the enhanced Sod2 expression observed during tumorigenesis, as p53 is one of the tumor suppressor genes frequently mutated and inactivated in a number of cancers. It should be noted that p53 may also regulate Sod2 activity independent of transcription. It has been shown that p53 can translocate to the mitochondria, following cellular stress signals such as treatment with the carcinogen 12-O-tetradecanoylphorbol-13-acetate (TPA)[60]. Here it can directly interact and inhibit activity of Sod2, which the authors show to be an important step in p53-mediated apoptosis following TPA treatment.

Oxygen free radicals and ionizing radiation have been shown to induce Sod2 expression in a TNFα-dependent manner, another transcription factor known to regulate transcription of Sod2 gene [61-63]. Changes in TNFα levels have been associated with enhanced metastatic disease [64,65]. A recent study suggests that the dichotomous expression of Sod2 in breast cancer might be explained by a novel transcriptional repressor DDB2 (damaged DNA binding 2), which leads to a loss of acetylated H3 histones and the decrease in Sp1 binding to the Sod2 promoter in Estrogen Receptor (ER) positive cells, like MCF-7 [66]. However,

in ER negative breast cancer cell lines, such as MDA-MB231, high levels of Sod2 have been proposed to be due to a lack of inhibition by this transcription factor [66]. In another breast cancer cell model, T47D, Sod2 expression has been shown to be under the influence of Progesterone [67].

Another possible explanation for the increase in Sod2 expression during tumorigenesis comes from a recent paper that shows a decrease in miRNA222 expression in an invasive squamous cell carcinoma cell line [68]. Interestingly this miRNA targets both Sod2 and MMP-1 for down regulation. We and others have shown that Sod2, via increased H₂O₂ can result in enhanced MMP-1 expression (see later chapter). Knockdown of miRNA 222 could therefore lead to an increase in MMP-1 via direct action on the MMP-1 gene and through indirect up regulation via Sod2 [68].

We and others are interested in the consequences of enhanced Sod2 during metastatic progression, and how this may influence intracellular ROS levels to manipulate pro-metastatic signaling events. While much of this research is still in its infancy we aim to provide evidence from recent studies to address the role of Sod2 and ROS during metastasis.

How does Sod2 influence tumorigenicity and metastatic progression?

The pioneering work of Oberley and Buettner opened a decade of enlightenment in the field of redox-biology and cancer. It was originally held that enforced Sod2 expression decreased proliferation of a wide array of tumor cells, both *in vitro* and *vivo*. These include human melanoma, glioma, prostate and breast cancer cell lines, SV-40 transformed fibrosarcoma cells and mouse fibrosarcoma and JB6 epithelial cells [69,70]. However, through the years the impact of molecular Sod2 enforcement on tumor progression has evolved. Clinically, studies have shown that tumors which display enhanced Sod2 expression are also less proliferative, but resistant to apoptosis [11,22] and in some cases no change in tumorigenesis is observed [71]. However, given the fact that Sod2 levels appear to increase during metastatic progression, we and others began to investigate the role Sod2 may play in signaling events that regulate metastatic spread. Using a fibrosarcoma cell model system Sod2 expression was shown to slow proliferation of HT1080 cells, with a concomitant increase in both the invasive and migratory phenotypes that is reversed by efficient H₂O₂ scavenging [72]. Changes in invasion are accompanied by changes in matrix metalloproteinase expression and matrix turnover and increases in metastatic lesions using the tail vein model metastases model [73]. In hamster cheek pouch cancer model Sod2 expression increased invasiveness that was partially abrogated by catalase co-expression [74]. We have also observed a similar phenotype in metastatic bladder cancer cells that is also tempered by catalase expression [36]. Removal of H₂O₂ also results in decreased clonogenic activity and VEGF expression [36]. Below we describe a number of pro-metastatic phenotypes that are driven by enforced Sod2 expression, and postulate on the potential mechanisms by which Sod2 may be regulating these events.

Sod2-mediated Mechanisms that regulate Metastasis

Matrix Metalloproteinases and Invasion—Matrix metalloproteinases (MMPs) are Zn²⁺ and Ca²⁺ dependent endopeptidases involved in extracellular matrix (ECM) turnover. To date, 23 known human MMPs have been identified; many of these share a five domain architecture comprising of a signal peptide, prodomain region, catalytic domain, hinge region and the hemopexin domain. Based upon their substrate specificity and structural differences, the MMPs are broadly classified into five groups – collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs (MT-MMPs). MMPs that differ from the general five-domain structure are: gelatinases (MMP-2 and 9), which have a fibronectin domain present within the catalytic domain; matrilysins (MMP-7 and 26), which lack the

hinge and hemopexin domain; and MT-MMPs that have a transmembrane domain and cytoplasmic tail. All MMPs are secreted as zymogens into the extracellular space with the exception of MT-MMPs, which have a furin recognition sequence at the end of prodomain region that allows for MT-MMPs intracellular activation [75].

In tissues, MMP levels are extremely low and strictly regulated due to their ability to cleave a wide variety of substrates as they modulate many physiological processes including wound healing, cellular growth, embryogenesis, immune surveillance and bone remodeling [76]. Elevated MMP levels have been associated with a variety of pathological conditions such as rheumatoid and osteo-arthritis, lung emphysema, atherosclerosis, Alzheimer's, and cancer. Overexpression of MMPs by tumor cells and/or stromal cells infiltrating the tumor can lead to the remodeling and degradation of the extracellular matrix and basement membranes, subsequent tumor cell invasion and metastasis[77]. Similarly, Sod2 mediated increases in cellular oxidant/anti-oxidant ratios are directly correlated with tumor progression, angiogenesis and migration and invasion. Zhang *et al.* (2002) demonstrated that overexpression of Sod2 in estrogen-dependent human breast cancer MCF-7 cells resulted in activation of MMP-2 expression and a corresponding increase in ROS [78]. Co-expression of catalase or glutathione peroxidase in the Sod2 overexpressing cells reversed this effect. Conversely, in the highly metastatic estrogen-independent breast cancer MDA-MB231 and SKBR3 cells, basal Sod2 levels are significantly higher as compared to the MCF-7 cells, with catalase and peroxiredoxin 3 expression significantly lower [79]. This augmented expression of Sod2 is associated with increased MMP-9 activity and greater invasiveness both of which are reduced by expression of anti-sense Sod2 RNA or treatment of the cells with the H₂O₂ scavengers NAC or pyruvate. These studies support that Sod2-mediated H₂O₂ production result in MMP dysregulation and subsequent metastasis.

Our group has demonstrated that overexpression of Sod2 in HT-1080 fibrosarcoma cells significantly enhanced migration and invasion both in vitro and in vivo [72]. The severity of invasion was directly correlated to the Sod2 levels; HT1080 cells expressing high levels of Sod2-GFP exhibit greater invasiveness, a loss of focal adhesions and an increase in lung metastasis as compared to cells expressing low levels of Sod2-GFP. In tumor cells isolated from pulmonary metastases, those that expressed high levels of Sod2-GFP exhibited greater motility when examined by time-lapse microscopy. Concomitant increases in MMP-1 levels that also correlated with Sod2 levels were observed. The dependence of MMP-1 expression and the metastatic phenotypes associated with high Sod2 expression were abrogated upon co-expression with catalase targeted to the mitochondria.

The ability of Sod2 overexpression to modulate invasion is not restricted to HT-1080 fibrosarcoma cells and was also observed in 253J transitional bladder carcinoma cells [72]. Similar to the HT-1080 fibrosarcoma cells, co-expression of catalase in the 253J bladder carcinoma cells reversed the severity of the invasive phenotype. The highly metastatic bladder cancer cell line 253J-BV, which was derived from the 253J-P parental cell line, exhibited enhanced expression and activity of Sod2 as compared to the parental cells [36,80]. The increase in Sod2 expression was accompanied by a significant decrease in catalase activity, resulting in a net increase in H₂O₂ production in the 253J-BV cell line. Expression of MMP-9 was upregulated in the metastatic line and was attenuated by overexpression of catalase. Similarly, expression of catalase effectively reduced the clonogenic activity of 253J-BV cells.

Mechanistically, the regulation of MMP-1 by Sod2 seems to be mediated in part at least, at the level of transcription [81-85,85]. Based upon promoter conformation, MMPs have been categorized into three groups [84]. MMPs in *group 1* have promoters that contain a TATA box in the -30 bp region with a proximal Activator Protein-1 (AP-1 site) at -70 bp (MMP-1,

3, 7, 9, 10, 12, 13, 19 and 26). Members in group 1 are transcriptionally activated following IL-1, TNF- α and phorbol ester treatment [86]. MMPs in *group 2* have a TATA box without an AP-1 binding site (MMP-8, 11 and 21) whereas MMPs in *group 3* lack all of the above elements (MMP-2, 14 and 28) [84]

Regulation of MMP-1 expression by Sod2 is influenced by a genetic variation in the MMP-1 promoter [73,82,83]. We have demonstrated that a single nucleotide polymorphism (SNP) located at -1607 bp, creates an Ets family transcription factor binding site of the sequence 5'-GGAT-3' by the insertion of a guanine base (G) and confers Sod2-dependent MMP-1 promoter activity [73]. In HT-1080 fibrosarcoma cells, Sod2 overexpression also increases the mRNA levels of MMPs -2, -3, -7, -10, -9, -11, however, we have not yet identified promoter elements responsible [73]. Ets transcription factors normally do not bind DNA alone, but preferentially form coactivator complexes with transcription factors, such as AP-1. The Ets and AP-1 coactivating complex may also play a critical role in regulating the expression of various MMP family members, particularly that of MMP-1 [87]. The protooncoproteins Fos and Jun, which comprise the AP-1 complex, can homo or heterodimerize and bind its cognate consensus sequence (TGACTCA) in the regulatory domains of many genes including various MMP family members [88].

Numerous reports indicate that transcription factors important for MMP-1 expression are sensitive to redox-activation that occurs, in part, through activation of the MAPK family members ERK1/2 and JNK [89-91]. We have established that JNK confers redox-sensitivity to the MMP-1 promoter while both ERK and/or JNK are required for maximal basal promoter activity and the expression of AP-1 and Ets-1 [82]. In addition, both c-Jun and Ets-1 are recruited to the region of the MMP-1 SNP in response to alterations in the steady state production of H₂O₂.

Other lines of evidence suggest that H₂O₂-generated by the Sod2 is important to enhanced expression of MMP-1. Scharffetter-Kochanek and co-workers demonstrated that overexpression of Sod2 in dermal fibroblasts caused a profound increase in the expression of MMP-1 when superoxide was provided to the cells [92]. The investigators established that the induction of MMP-1 is due to the increase in the production of H₂O₂ as a result of the dismuting function of Sod2. This is further supported by work from our group that indicates binding of Ets-1 to the MMP-1 promoter and sustained JNK signaling requires the enzyme activity of Sod2. Furthermore, Sod2 is sufficient and required for the induction of MMP-1 and potentially other MMP family members via the H₂O₂-dependent activation of MAPK signaling [73,81,82].

Redox sensitive metastatic signaling networks: regulation of phosphatases—

While we have extensively shown that enhanced expression of Sod2 leads to changes in MMP expression, our lab is interested in the mechanisms by which Sod2 expression can influence other pro-metastatic signaling event, potentially as a consequence of changing the redox environment of metastatic cancer cells. While Sod2 expression has been characterized to inhibit proliferation in some tumor cell types, an Sod2-dependent change in redox state appears to promote pro-migratory and -invasive signaling events.

ROS can act as second messengers via the oxidation and inactivation of Phosphatases. The active site cysteine residues responsible for phosphoryl transfer are particularly susceptible to oxidation at physiological pH, due to their low pKa (For review see [93]). This oxidation is reversible and dependent on the thiol status of cells and can result in significant changes in cellular signaling.

PTEN (Phosphatase and Tensin Homolog) is a dual protein and lipid phosphatase that has been extensively studied as a tumor suppressor gene. Loss or functional mutants of PTEN are associated with a number of cancers and poor prognosis [94]. The lipid phosphatase activity of PTEN results in dephosphorylation of PtdIns(3,4,5)P3 to PtdIns(4,5)P2, essentially regulating the phosphoinositide 3-kinase (PI3) Kinase signaling pathway by limiting PtdIns(3,4,5)P3 levels. PtdIns(3,4,5)P3 is an important regulatory lipid, functions of which range from involvement in migration to angiogenesis. Redox dependent changes of the PI3-kinase pathway have been attributed to the reversible oxidation and inactivation of PTEN [95]. We have found that HT-1080 fibrosarcoma cells that express Sod2 display more oxidation of this phosphatase than control transfected cells [96]. Further, oxidation of PTEN can be reversed by catalase expression, potentially implicating an increase in H₂O₂ as the primary ROS involved. PTEN oxidation is enhanced in the metastatic cell line, 253J-BV, which displays an increase in endogenous Sod2 expression, low catalase expression and an accompanying increase in H₂O₂ levels, when compared to its non-metastatic parental line 253J-P (Hempel, Ye and Melendez, unpublished data). In the HT-1080 Sod2 over-expression model enhanced PTEN oxidation leads to a redistribution of PtdIns(3,4,5)P3 to the membrane, and leads to enhanced Akt signaling and increased angiogenesis, which were all Sod2 dependent [96].

We and others are actively investigating how increases in mitochondrial Sod2 and potential shifts in steady state [H₂O₂] lead to oxidation of phosphatases in membrane and cytosolic compartments. While largely unknown, possibilities include the ability of oxidants to diffuse from the mitochondria to broad areas within the cell and/or the physical distribution of mitochondria. Mitochondria are often localized to leading edges of migrating cells, presumably to provide an energy source. Whether this also reflects site specific increases in mitochondrial ROS at the membrane remains to be elucidated. The importance of oxidants influencing cellular distribution of signaling players is highlighted by preliminary data from our laboratory. We have found that the oxidized pool of PTEN is recruited away from the membrane, its primary site of action, and more abundantly found throughout the cytosol (Hempel and Melendez, unpublished data). This suggests that oxidation not only leads to a decrease in activity, but a change in cellular localization of this phosphatase.

Enhanced PtdIns(3,4,5)P3 distribution to the leading edge of cells has been implicated in regulating directionality and migration [97]. It is possible that cell surface accumulation of PtdIns(3,4,5)P3 in Sod2 over-expressing cells contributes to their enhanced migratory phenotype. Evidence from PTEN mutagenesis studies however suggest that some of the metastatic regulatory properties of PTEN may not be solely due to its lipid phosphatase activity. Leslie and colleagues have shown that the protein tyrosine phosphatase activity of PTEN negatively regulates epithelial to mesenchymal (EMT) transition [98]. Whether shifts in the Sod2-mediated oxidation of PTEN also drive EMT remains to be established. However, evidence from TGF- β induced EMT in human renal epithelial cells, suggests that Sod2 expression is down regulated during this transition [99]. Nonetheless, Sod2-mediated redox shift enhances Akt signaling. Sod2 expressing cells increase their angiogenic activity, a key contributor to metastatic spread and tumor growth [96]. Enhanced angiogenesis was accompanied by Sod2-dependent increases in VEGF expression [96]. The Sod2-dependent angiogenic switch was reversed by catalase expression, suggesting a role for H₂O₂ in this process. In 253J-BV cells, which display endogenously high Sod2 levels, we have similarly shown that enhanced VEGF levels and MMP-9 levels are dependent on a high intracellular H₂O₂ milieu [36]. Again, catalase expression could abrogate expression of these pro-metastatic genes.

Other phosphatases involved in pro-migratory signaling have similarly been shown to be redox regulated. We have investigated the role of Sod2 expression on changes in Focal

Adhesion Kinase (FAK) signaling and found that the FAK-signaling cascade is activated in cells with high Sod2 expression. FAK signaling has also been shown to be H₂O₂-dependent, as catalase co-expression could reverse this effect, mediated via oxidation of phosphatase PTPN12 (Hempel and Melendez, unpublished data). Similar to Sod2, expression of Lysyl oxidase (LOX) has been shown to induce migration in a H₂O₂ dependent manner [100]. It was shown that FAK and Src activation were increased in LOX transfected breast cancer cells and catalase could abrogate the effects on migration and phosphorylation of the pro-migratory signaling kinases.

Other phosphatases that may be regulated due to a Sod2-dependent altered redox status likely include the Map Kinase phosphatases (MKP) that regulate MAPK signaling. MKPs are similarly sensitive to redox-dependent inactivation and this oxidation participates in the senescence-associated increase in MMP-1 expression [101]. MKPs are known targets of oxidation which leads to enhanced proteasomal degradation [102]. It is exciting to speculate that a similar signaling network participates in the Sod2-mediated increase in MMP expression in tumor cells.

Protection from Apoptosis—Many studies have shown that Sod2 is an important enzyme that protects cells from radiotherapy induced apoptosis. Here the action of Sod2 has primarily been attributed to its antioxidant activity, rather than shifting the redox balance to a pro-H₂O₂ state. It has been shown that Sod2 stabilizes the mitochondrial membrane and leads to decreased caspase3 activation when cancer cells are treated with ionizing radiation, compared to cells that lack high Sod2 expression [103]. In addition Sod2 may inhibit tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by inhibiting cytochrome c and Smac/DIABLO release [104]. The authors hypothesize that within metastatic tumor cells the high levels of mitochondrial ROS, which normally drive release of Smac/DIABLO, are neutralized by Sod2, to prevent apoptosis. The high levels of Sod2 in tumor cells are therefore detrimental to chemo- and radiation therapy and investigators have proposed the use of Sod2 inhibitors as adjuvant therapy to radiation.

Wong and Goeddel were first to report that Sod2 was induced in response to tumor necrosis factor (TNF) and interleukin-1, as a protective mechanism from cytokine induced cell death [105,106]. Enforced Sod2 expression has been shown to confer resistance to numerous apoptotic stimuli [107-110]. The antiapoptotic effects of Sod2 are attributed to its dismuting activity. Recent work suggests that increases in steady state [H₂O₂] resulting from the overexpression of Sod2 are responsible for restricting TNF induced apoptotic cell death [111]. Regardless, of mechanism of protection afforded by Sod2 overexpression it does not precisely fit its role as a tumor suppressor as it would serve to prolong tumor survival and resistance to chemotherapeutic strategies. Recent work indicates radiation-resistance of cancer stem cells is attributed to their increased free radical scavenging capacity [112]. Chemoresistance of urothelial carcinoma cancer stem cells is associated with a high level expression of Sod2 [113,114]. Enhanced ROS scavenging confers genoprotection and allows cancer stem cells the unique ability to colonize new sites and survive after chemo- or radiation therapy. Thus, increased Sod2 expression may promote cancer stem cell survival and subsequent metastases.

Conclusions—It has been more than 30 years since Oberley and Buettner made the connection between low enzymatic Sod2 activity and cancer [1]. The idea that loss of Sod2 would potentiate tumorigenesis was well founded principally due to the wealth of information linking oxidants to tumor promotion [115]. This early observation has stood the test of time and it is now clear that in many tumor types suppression of Sod2 is regulated at both the genetic and epigenetic level [54,116]. While loss of Sod2 sensitizes tumor cells to free radicals associated with both radiation and chemotherapeutic it is equally not surprising

that metastatic cancers that resist traditional ionizing and chemotherapies display an increase in dismuting capacity. Why this increase is primarily restricted to Sod2 is not clear? Sod2 serves to preserve mitochondrial integrity but this is not likely the case as loss of mitochondrial genome integrity precedes progression to metastatic disease and is associated with increased ROS production [117]. Thus, increases in Sod2 levels may help cope with increased metabolic oxidant production. Alternatively, metastatic tumor cells are continuously exposed to inflammatory stimuli which can drive Sod2 expression. While possible, this does not explain the high level expression of Sod2 in isolated metastatic tumor cell types. Thus, many metastatic tumor cell types are programmed for high level Sod2 expression independent of inducing stimuli. There may be countless reasons for the increased Sod2 that is associated with a diverse array of malignant lesions and metastatic tumor cell lines. We feel it is the resulting consequences of increased Sod2 expression that further propagate the malignant phenotype as discussed in detail on the preceding pages and outlined in Figure 2. So far the pro-metastatic profile associated with increases in Sod2 expression has been attributed to increases in steady state $[H_2O_2]$. H_2O_2 can restrict the activity of many protein tyrosine phosphatase family members leading to an inherent increase in basal kinase activity. Our studies have established that Sod2-mediated increases in $[H_2O_2]$ lead to oxidative inactivation of PTEN, Map kinase phosphatases and PTPN12 that play an important role in restricting invasion/migration, matrix turnover, cell proliferation, angiogenesis and cell survival. It is likely that elevated steady state $[H_2O_2]$ resulting from Sod2 impact a broad array of signaling networks all of which exacerbate the malignant phenotype. The fact that many or all of these malignant properties are reversed by catalase co-expression makes them amenable to targeted and precise antioxidant intervention.

Interestingly, Oncomine data mining revealed that catalase levels are often decreased in a number of cancer types and expression appears to decrease further with progression to metastatic disease (Oncomine.org)[118]. Catalase levels are negatively regulated by a number of mechanisms, including oxidant dependent promoter hypermethylation [119], phosphorylation and consequential proteosomal degradation[120]. One could speculate that an increase in Sod2 activity and a concomitant decrease in catalase activity may further shift the steady state level of $[H_2O_2]$ within tumor cells.

Recent studies have shown that Nox4 levels are increased in tumors of the breast, ovary and melanoma, and that this enhanced expression leads to an increased tumorigenic phenotype[121,122]. Further, Graham *et al.* reported that this enhanced Nox4 dependent tumorigenicity is dependent on Nox4 localization to the mitochondria and a concomitant increase in mitochondrial ROS production [121]. Whether an increase in mitochondrial Nox4 levels within metastatic tumor cells provides the source for enhanced $O_2^{\cdot-}$ production, which are consequentially rapidly dismutated by Sod2 within these cells, thereby resulting in a change in $[H_2O_2]$ levels, remains to be elucidated.

While we have experimental evidence indicating that steady state $[H_2O_2]$ levels increase in response to Sod2 expression, the exact biochemical rationale for this shift is beyond the scope of this review and we refer the reader to reviews by Dr. Gary Buettner and Dr. Irwin Fridovich on this topic

The differences in pro- and anti-oxidant activity of Sod2 may also be related to dose dependent effects of Sod2 expression. For example, Wang *et al.* reported that Sod2 has a biphasic role on Hypoxia Inducible factor-1 α (HIF-1 α) regulation. Low level increases in Sod2 expression (2-6-fold) in MCF-7 breast cancer cells suppressed HIF-1 α accumulation under hypoxic conditions[123]. The same authors later showed that this suppression was due to the dismuting activity of Sod2, as accumulation of $O_2^{\cdot-}$ following Sod2 knock-down was

able to enhance HIF-1 α levels[124]. On the contrary, when Sod2 was expressed at levels higher than 6-fold, HIF-1 α levels increased in a H₂O₂ dependent manner, as peroxide removal from the mitochondria could reverse this effect[125]. We did not observe these biphasic effects of Sod2 expression on migration of HT-1080 cells. Low levels of Sod2 expression (2.5-fold increase in Sod2 activity) were able to drive migration of HT-1080 cells and additional increases in Sod2 expression lead to further enhancement of this migratory phenotype[126]. However, we have observed a biphasic role for Sod2 in the expression of the cytokine IL1 α . At low Sod2 concentrations IL1 α transcript levels are decreased [127], yet at higher Sod2 levels IL1 α expression is H₂O₂-dependent.

There is a need to further our understanding of these complex biochemical mechanisms that govern the pro-oxidative and anti-oxidative nature of Sod2 during tumorigenesis. This knowledge will shed light on the roles of different oxidant species within tumor cells, as well as the consequences of altering antioxidant enzyme levels such as Sod2. It will also aid in the development of more targeted antioxidant and Sod2 mimetic therapies, considering that certain Sod2 mimetics may exert both pro- and anti-oxidative actions within tumor cells (review by Ines Batinic-Haberle in this issue). In addition, it is important to keep in mind that cancer stem cells bolster their free radical scavenging capacity for survival. Thus, it is not surprising that nutritional antioxidant supplementation has failed in cancer prevention studies as they have broad ranging impact and serve to scavenge all oxidants regardless of whether they are detrimental or beneficial to tumor cell survival. The advent of specific synthetic free radical scavengers which do not give rise to free radical propagating species may hold great promise in treatments targeted at metastatic disease.

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Figure 1.

Changes in Sod2 mRNA levels are associated with Grade, Metastatic Progression and Survival of Brain Cancer (A, [128], ***p<0.001, t-test; B, [9], p<0.05, t-test); Prostate Cancer (C,[129], *p<0.05, *** p<0.001, ANOVA, Tukey's Post test) and Breast Cancer (D. [130], *p<0.05, *** p<0.001, ANOVA, Tukey's Post test; E. [131], **p<0.01, t-test). Data was obtained from oncomine.org. Boxes represent the interquartile range marking the 25th to 75th percentile, midline indicates the median, whiskers mark the 10th and 90th percentile. Statistical Analysis was performed using GraphPadPrism software.

Figure 2. Pro-metastatic signaling pathways regulated by Sod2

Enhanced expression of Sod2 in tumor cells have been linked with increased expression and activity of Matrix Metalloproteinases, leading to enhanced matrix degradation and potentially enhanced release of cytokines and growth factors, which further enhance signaling via kinases. ECM degradation aids a tumor cell's ability to leave the primary tumor and invade through the basement membrane. Enhanced H_2O_2 production following Sod2 expression has been linked to increased angiogenesis and migration, via the inactivation of protein tyrosine phosphatases (PTPs), subsequently enhancing kinase signaling, such as the PI3Kinase and FAK signaling cascades. Enhanced Sod2 expression has been directly linked to tumor cell radio and chemo-resistance, likely due to its dismuting activity, however it is possible that this may be further enhanced via Sod2's role in regulation of redox signaling.

Table 1

Changes in Sod2 mRNA levels from microarray studies of tumor specimens.

Cancer Type	Number of studies reporting changes in Sod2 levels	
	Increase	Decrease
Kidney	9	-
Brain & CNS	7	-
Head & Neck	7	-
Colorectal	5	-
Lymphoma	5	-
Testicular Seminoma and Teratoma	4	-
Gastric	3	-
Squamous Cell Skin Carcinoma	2	-
Pancreatic	2	-
Ovarian	1	-
Cervical	1	-
Esophageal	1	-
Liver	1	1
Breast Cancer	1	2
Other *	1	2
Sarcoma	3	2
Bladder Cancer	-	1
Prostate	-	2
Leukemia	1	5
Lung	-	-
Melanoma	-	-
Myeloma	-	-

Data was derived from a data base search on Oncomine.org, using a threshold p-value of <0.01 and >2-fold change in mRNA expression. – indicates no significant changes in Sod2 levels.

* Other Cancers include: increase in Malignant Fibrous Histiocytoma, and decrease in Testicular Intratubular Germ Cell Neoplasia and Testicular Yolk Sac Tumor.