Mitochondrial Superoxide Dismutase Has a Protumorigenic Role in Ovarian Clear Cell Carcinoma

L.P. Madhubhani P. Hemachandra, Dong-Hui Shin, Usawadee Dier, James N. Iuliano, Sarah A. Engelberith, Larissa M. Uusitalo, Susan K. Murphy, and Nadine Hempel

Abstract

Epithelial ovarian cancer (EOC) is the fourth leading cause of death due to cancer in women and comprises distinct histologic subtypes, which vary widely in their genetic profiles and tissues of origin. It is therefore imperative to understand the etiology of these distinct diseases. Ovarian clear cell carcinoma (OCCC), a very aggressive subtype, comprises >10% of EOCs. In the present study, we show that mitochondrial superoxide dismutase (Sod2) is highly expressed in OCCC compared with other EOC subtypes. Sod2 is an antioxidant enzyme that converts highly reactive superoxide (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$) and oxygen (O$_2$), and our data demonstrate that Sod2 is protumorigenic and prometastatic in OCCC. Inhibiting Sod2 expression reduces OCCC ES-2 cell tumor growth and metastasis in a chorioallantoic membrane (CAM) model. Similarly, cell proliferation, migration, spheroid attachment and outgrowth on collagen, and Akt phosphorylation are significantly decreased with reduced expression of Sod2. Mechanistically, we show that Sod2 has a dual function in supporting OCCC tumorigenicity and metastatic spread. First, Sod2 maintains highly functional mitochondria, by scavenging O$_2^-$, to support the high metabolic activity of OCCC. Second, Sod2 alters the steady-state ROS balance to drive H$_2$O$_2$-mediated migration. While this higher steady-state H$_2$O$_2$ drives prometastatic behavior, it also presents a doubled-edged sword for OCCC, as it pushed the intracellular H$_2$O$_2$ threshold to enable more rapid killing by exogenous sources of H$_2$O$_2$. Understanding the complex interaction of antioxidants and ROS may provide novel therapeutic strategies to pursue for the treatment of this histologic EOC subtype.

Introduction

Ovarian clear cell carcinomas (OCCC) represent approximately 10% to 25% of all epithelial ovarian cancer (EOC), depending on ethnic background (1). It is now evident that OCCC differs widely from the more common high-grade serous adenocarcinoma. While the primary tumor mass of OCCC is found on the ovary, its origin is not thought to be the ovary or fallopian tube but rather to stem from endometrioid tissue and endometriosis. Because of its origin is not thought to be the ovary or fallopian tube but rather to stem from endometrioid tissue and endometriosis. Ovarian clear cell carcinoma (OCCC), a very aggressive subtype, comprises >10% of EOCs. In the present study, we show that mitochondrial superoxide dismutase (Sod2) is highly expressed in OCCC compared with other EOC subtypes.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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high mitochondrial function and proliferation, but also alters the steady-state ROS balance to drive H2O2-mediated migration and metastasis of OCCC cells.

Materials and Methods

Oncomine data and ovarian cancer cell line microarray data

Oncomine.org was used to screen Sod2 expression in ovarian cancer histologic subtypes (Supplementary Fig. S1). Two representative datasets are shown in Fig. 1A and B (GEO accession nos. GSE2109 and GSE6008). Microarray data of the following ovarian cancer cell lines were obtained using the GeneChip Human Genome U133A 2.0 Array (Affymetrix; GEO accession no.: GSE25428; refs. 4, 11). Data represent expression of Sod2 probe 215223_s_at (log2 RMA normalized). OCCC: JHOC-5, JHOC-7, JHOC-8, JHOC-9, KOC-5C, KOC-7C, OVISE, OVTOKO, RMG-1, RMG-2, RMG-5, TAYA, TOV-21-G. Serous adenocarcinoma: CAOV3, Fuov1, HEY, Hey-A8, Hey-CE, JHOS-2, JHOS-3, JHOS-4, M41, M41-cisR, OV90, OVARY1847, OVCA420, OVCA429, OVCA432, OVCA3, PE01, PE04, SKOV3. Mucinous: JHOM-1, JHOM-2B, MCAS, OMC-3. Endometrioid: OVK-18, TOV-112D. Adenocarcinoma: A2780 (A2780-J), A2780-cisR, DOV13, OVCA2, OVCA5, OVCA8. Teratocarcinoma: CH1, PA1. Undifferentiated: TYK-nu, TYK-nu cisR. Prior to microarray analysis, cell lines were authenticated by STR analysis at the Fragment Analysis Facility, Johns Hopkins University (Baltimore, MD; PowerPlex 1.2 System; Promega) or at the University of Colorado Cancer Center (Aurora, CO; AmpFlSTR Identifier Plus PCR Kit, Applied Biosystems; ref. 11).

Cell lines and cell culture conditions

At commencement of this study ES-2 and TOV-21-G cells were newly obtained from ATCC. Authenticity was verified by ATCC using STR analysis. ES-2 cells were maintained in McCoy 5A media + 10% FBS and TOV-21-G cells in 40% Media199/40% MCBD supplemented with 20% FBS and sodium bicarbonate. Cells were maintained at 37°C with 5% CO2.

Sod2 knockdown using RNA interference

Scramble nontargeting control and Sod2-specific siRNA oligonucleotides were synthesized by Life Technologies/Dharmacon. 5'-CAACAGGCCUUAUUCCACU-3' and 5'-AAGUAAACCAC-GAUCGUUA-3' sequences were used as siSod2_#1 and siSod2_#2, respectively (Supplementary Fig. S2) and 10 pmol transfected into cells using Lipofectamine RNAiMax (Invitrogen).
shRNA with nontargeting scramble sequence or targeting Sod2 (shSod2_#1: 5'-CTGACGGCTGCATCTGTTGGTGTCCAAGG-3', and shSod2_#2: 5'-ACCTGAACGTCACCGAGGAGAAGTAC-3') in pGFP-V-RS vector (Origene; TG309190) were used to stably transfect ES-2 cells (Fig. 2; Supplementary Fig. S3). The clone expressing shSod2_#1 was used in Figs. 2–7.

**Immunoblotting**

Protein expression was analyzed by standard Western blotting using antibodies from Cell Signaling Technology (pAkt-s473, Akt, pFAK-Y397, FAK, p-p130cas-Y165, p130cas) or Abcam (Sod2). Primary antibodies were diluted in blocking solution (5% nonfat milk in TBS with 0.1% Tween-20, 1:1,000) and incubated overnight at 4°C. Blots were visualized using Femto and Pico ECL chemiluminescence substrate (Thermo scientific) and imaged using a ChemiDoc MP system (BioRad). Densitometric analysis was performed using ImageJ software (NIH). Each protein band was normalized to the respective GAPDH or β-actin loading control band.

**Sod2 zymography**

Sod2 activity was analyzed using Sod2 in-gel zymography as previously described (12). Briefly, cell lysates were loaded on nondenaturing acrylamide gels, followed by electrophoresis. Sod2 activity is visualized by the inhibition of nitroblue tetrazolium reduction.

**Clonogenicity and cell viability**

Single-cell survival clonogenicity assays were performed as previously described (13). Briefly, 100 cells were plated in each well of 6-well plate colonies visualized after 10 days using crystal violet. Viability was assessed by cell counting using trypan blue (1%) staining or crystal violet uptake assays (13).

**Chorioallantoic membrane assay**

Each chorioallantoic membrane (CAM) was inoculated with 5 × 10^5 ES-2 cells stably expressing either scramble-shRNA-GFP or Sod2-shRNA_#1-GFP that were suspended in 50 μL PBS (with 1 mmol/L MgCl_2, 0.5 mmol/L CaCl_2, 100 U/mL penicillin, and 100 μg/mL streptomycin), essentially as previously described (14). Tumors were allowed to form for 7 days prior to termination of the experiments by sacrificing the chick embryo. Tumors on the CAM were removed and measured. CAM and chick embryo organs (liver and lung) were collected for tumor metastasis analysis by surveying for GFP-labeled cells.

**Seahorse XF24 extracellular flux analysis**

Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and mitochondria stress tests were measured using the Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience), as described previously (13). Cells were plated at a density of 40,000 cells per well and media replaced with XF media the following day 1 hour prior to the assay. Three measurements of OCR and ECAR were taken at baseline and after each injection of the following mitochondrial stress test compounds: oligomycin (1 μmol/L; complex V inhibitor); FCCP (0.75 μmol/L; proton gradient uncoupler); and antimycin A (1 μmol/L; complex III inhibitor). Basal and maximal respiration were normalized by subtracting nonmitochondrial OCR (i.e., after antimycin A addition). Respiratory reserve capacity was calculated as the difference between basal and maximal OCR. ATP-linked OCR...
was derived as the difference between basal and oligomycin A–inhibited OCR. Data were normalized to total protein content in each well.

Wound-healing assay
Cell migration was assessed in serum-free media by wound-healing assays using Ibidi inserts (Martinsried) and quantified after 72 hours. Ibidi inserts were removed from a monolayer of GFP-labeled cells to expose the cell-free wound area. Fluorescent images were taken after 72 hours of migration and overlayed with corresponding images at time 0 hour. Pixels representing GFP-labeled cells were quantified within the wound area using ImageJ and corrected by subtracting any GFP-detected cells in the same area at time 0.

Spheroid attachment assay
Cells were plated at a density of 1,000 cells per well in ultra-low attachment 96-well plates (Corning) and incubated for 5 days. Spheroids were transferred to 24-well plates with or without Collagen 1 coating. Percentage outgrowth was calculated by

Figure 3. Sod2 preserves OCC mitochondrial respiration. A, reduced Sod2 levels significantly attenuated the mitochondrial oxidative phosphorylation in stably transfected ES-2 cells (shSod2_#1). OCR was measured using extracellular flux analysis and pharmacologic manipulation of mitochondrial activity to interrogate bioenergetics parameters. Oligomycin A (O) was used to derive ATP-linked OCR, FCCP (F) to stimulate maximal OCR, and antimycin A (A) to inhibit all mitochondrial OCR. B, basal OCR, ATP-linked OCR, maximum OCR, and respiratory reserve capacity (max OCR – basal OCR) were significantly decreased with stable shRNA-mediated Sod2 knockdown in ES-2 (one representative of three experiments is shown; mean ± SEM; control, n = 8; shSod2, n = 9. ANOVA, Tukey posttest: **P < 0.001; ***P < 0.0001). C, transiently transfected siRNA targeting Sod2 in TOV-21-G cells showed significantly reduced mitochondrial oxidative phosphorylation (siRNA construct #1). D, basal OCR, maximum OCR, and respiratory reserve capacity (max OCR – basal OCR) were significantly decreased with Sod2 knockdown in TOV-21-G cells (one representative of three experiments is shown; mean ± SEM; control, n = 4; siSod2, n = 5; ANOVA, Tukey posttest; *P < 0.05). E, superoxide-mediated oxidation of MitoSox redox-sensitive dye was measured in live cells. Increased MitoSox fluorescence was observed in ES-2 cells stably transfected with shRNA-Sod2_#1 compared with scramble-transected control cells, suggesting that the amount of mitochondrial superoxide accumulation is higher in cells with reduced Sod2 expression. MitoSox fluorescence was abrogated by addition of 10 μmol/L of the porphyrin superoxide scavenger ortho tetrakis(N-n-butoxyethylpyridinium-2-y) porphyrin (MnTnBuOE-2-PyP5+), representative of three experiments is shown; mean ± SEM, n = 4; ANOVA, ANOVA Tukey posttest; *P < 0.05, **P < 0.01, ***P < 0.001.

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Reduced Sod2 levels significantly inhibit OCCC cell proliferation in vitro and tumor formation in the CAM model

To further investigate the role of Sod2, we used two OCCC cell lines, ES-2 and TOV-21-G. Sod2 expression was inhibited by shRNA and siRNA transfection, which was demonstrated to lead to a concomitant decrease in Sod2 enzyme activity (Fig. 2A; Supplementary Figs. S2 and S3A). Following Sod2 expression knockdown, ES-2 cell proliferation rate (Fig. 2B; Supplementary Fig. S3B) and clonogenicity (Fig. 2C; Supplementary Fig. S3C) were significantly attenuated. This appeared to be Sod2 concentration-dependent. A 30% reduction in Sod2 levels mediated by stable shRNA transfection reduced clonogenicity by approximately 50% (shSod2_#1), whereas a 70% Sod2 decrease almost completely abrogated the cells' ability to survive in this assay (shSod2_#2; Supplementary Fig. S3C). Analysis of PARP cleavage and Annexin V staining suggested that the decrease in cell viability observed in cells with 30% Sod2 knockdown is not related to a significant increase in apoptosis (Supplementary Fig. S4). This cell line, referred to as shSod2 in the following figures, was chosen for subsequent studies to achieve pathophysiologically relevant changes in Sod2 expression (rather than complete loss), which also closely reflects Sod2 levels observed in non-OCCC cell lines OVCA433 and OVCA429 (Fig. 1E). The CAM ex vivo model was used to further test the role of Sod2 on ES-2 tumorigenicity. A significant decrease in tumor size and weight was observed in tumors grown from ES-2 cells with reduced Sod2 expression (shSod2; Fig. 2D). In addition, the shRNA-Sod2 tumors exhibited less vascularization than the control groups (Fig. 2D), suggesting that Sod2 may contribute to both proliferation and the recruitment of blood vessels to the tumor.

Sod2 maintains OCCC mitochondrial respiration

We previously demonstrated that OCCC cell lines are highly energetic and depend on both oxidative phosphorylation and glycolysis for their energy needs (13). Given that Sod2 has a primary role in protecting mitochondria from excess O$_2^-$, the effect of Sod2 knockdown on mitochondrial respiration was assessed. OCR, representing mitochondrial oxidative phosphorylation, and ECAR, correlating with glycolytic activity, were measured using extracellular flux analysis in both ES-2 and TOV-21-G OCCC cell lines following Sod2 knockdown (13, 16). Stable shRNA-Sod2 decreases in ES-2 and transient siRNA-mediated knockdown of Sod2 in TOV-21-G cells resulted in significant reduction in basal OCR compared with control scramble RNAi–transfected cells (Fig. 3A–D; Supplementary Fig. S5A). Furthermore, respiratory reserve capacity, a measure of the ability of cells to enhance respiration in response to physiologic cues and stress, was significantly inhibited with reduced Sod2 levels (Fig. 3B and D), suggesting that Sod2 plays a major role in maintaining mitochondrial health to support maximal respiration. Although slight, a consistent negative effect on OCR was observed in stable shRNA-Sod2 cells following FCCP treatment (Fig. 3B). A wide range of FCCP concentrations was tested on these cells, but none were able to enhance OCR with Sod2 loss. FCCP can be inhibitory at high concentrations, and it has been speculated that this may be due to a loss in the ability of mitochondria to accumulate respiratory substrates (16). While not tested here, it is possible that sustained Sod2 expression decreases, and concomitant increases in mitochondrial O$_2^-$ levels, may exacerbate this FCCP-dependent OCR inhibition, thereby influencing mitochondrial membrane integrity and substrate transport. No significant increases in ECAR were observed between control and shRNA- or siRNA-transfected cells, suggesting that decreases in Sod2 expression do not influence a compensatory shift toward glycolysis (Supplementary Fig. S5B–SD). The above observations show that both siRNA- and shRNA-mediated decreases in Sod2...
reduced basal OCR and respiratory reserve capacity, indicating that Sod2 is important in maintaining mitochondrial respiration in OCCC.

To assess whether a decrease in Sod2 expression results in compromised \( \text{O}_2^- \) scavenging, which may be one of the causes of compromised mitochondrial function, the presence of mitochondrial \( \text{O}_2^- \) was evaluated using the mitochondria-targeted redox-sensitive dye MitoSox. As expected, increased oxidation and consequential enhanced fluorescence of MitoSox were observed in the Sod2-knockdown cells compared with controls (Fig. 3E and F). Addition of the Sod2 mimetic porphyrin (MnTnBuOE-2-PyP5+), which acts as a \( \text{O}_2^- \) scavenger, reduced MitoSox oxidation in both control and shRNA-Sod2 groups (Fig. 3E and F).

**Sod2 knockdown attenuates metastasis of cancer cells in the CAM model**

We have previously demonstrated that enhanced Sod2 expression is implicated with metastatic progression (7, 8, 17). To investigate the role of Sod2 during OCCC metastasis, the appearance of metastatic lesions of GFP-labeled ES-2 cells was investigated in the CAM tumor model. Single cells and micrometer-sized cellular clusters were highly abundant throughout the membrane in the control group, which could be observed 2 to 3 cm from the tumor (Fig. 4A). In contrast, metastatic spread from shRNA-Sod2 knockdown tumors was limited to the appearance of single cells in the membrane confined to an approximate 1- to 1.5-cm radius from the tumor (Fig. 4A). Furthermore, lung metastases in the chick embryo were observed in 11 of the 12 controls compared with only 5 of 10 embryos in the shRNA-Sod2 group (Fig. 4B). Furthermore, 10 of 12 control tumors metastasized into the liver, whereas only 4 of 10 liver metastases were observed in the shRNA-Sod2 group (Fig. 4B). While clusters of five or more cells were found in the lungs and livers of control groups, only single cells were detected in the Sod2-knockdown group (Fig. 4B).

**Sod2 levels modulate cell migration and tumor spheroid outgrowth**

Because of the significant abrogation of metastatic spread in response to Sod2 expression decreases, the role of Sod2 on cell migration was further investigated. Cell migration, assessed by wound-healing assays, was significantly inhibited with reduced Sod2 expression in ES-2 cells (Fig. 5A; Supplementary Fig. S6). Furthermore, the ability of cellular spheroid clusters to...
attach and cells to migrate from the spheroid onto collagen I and uncoated surfaces was also compromised with reduced Sod2 expression (Fig. 5B). Anchorage-independent spheroid formation is a commonly observed phenotype of ovarian cancer cells metastasizing via the transcoelomic route through the intraperitoneal cavity, and these have shown the ability to attach on the peritoneum to form metastatic lesions. These data suggest that Sod2 plays an important role in tumor spheroid metastasis (Fig. 5B). While spheroids of equal size were chosen for this assay, it should be noted that Sod2 knockdown also decreased ES-2 spheroid growth in anchorage independence (data not shown).
To gain mechanistic insights into the signaling pathways that may be altered by Sod2-mediated metastasis, phosphorylation profiles of Akt, p130cas, and focal adhesion kinase (FAK) were investigated. These were chosen on the basis of previous observations of their redox regulation and involvement in tumor cell migration (8, 9, 18, 19). shRNA-Sod2 cells exhibited a 50% decrease in phospho-Akt levels compared with scramble control cells, whereas no appreciable change was observed in phosphorylation of FAK or the focal adhesion adapter protein p130cas (Fig. 5C). The effects on Akt phosphorylation were also dependent on Sod2 concentration, where cells with lower Sod2 expression demonstrated a more striking decrease in Akt phosphorylation...
(Supplementary Fig. S6). These data suggest that Akt signaling may be important in driving Sod2-mediated tumorigenicity and metastasis of OCCC.

**OCCC cell migration is H$_2$O$_2$-dependent**

Sod2 is the primary enzyme involved in converting O$_2^-$ to H$_2$O$_2$ within the mitochondria. While it serves as a protective mechanism to maintain mitochondrial function (Fig. 3) by removal of damaging O$_2^-$, a shift toward increasing levels of H$_2$O$_2$ has also been observed in response to enhanced Sod2 expression (8, 10, 18, 20, 21). Because of its relative stability and ease in traversing cellular membranes, H$_2$O$_2$ can mediate redox signaling, including events that drive migration (9). To test whether Sod2 changes the steady-state H$_2$O$_2$ levels in OCCC, we assessed intracellular H$_2$O$_2$ status in control and shRNA-Sod2 ES-2 cells using a biochemical assay on the basis of the irreversible inhibition of catalase by aminotriazole (15, 17). Steady-state levels of H$_2$O$_2$ were reduced approximately by 50% in Sod2-knockdown cells compared with controls (Fig. 6A), whereas baseline catalase activity and protein expression were comparable (Fig. 6B).

To test whether OCCC cell migration is H$_2$O$_2$-dependent, wound-healing assays were carried out in the presence of catalase, which catalyzes the conversion of H$_2$O$_2$ to H$_2$O and O$_2$. As previously demonstrated, exogenous application of recombinant catalase resulted in accumulation of catalase within ES-2 cells (Supplementary Fig. S7; ref. 9). Catalase significantly reduced the migration of both control and shRNA-Sod2 cells, suggesting that H$_2$O$_2$ is a promoter of ES-2 cell migration (Fig. 6C). Conversely, treatment with low levels of H$_2$O$_2$ (5 µmol/L) significantly reversed the slow migration of shRNA-Sod2 cells (Fig. 6C). Furthermore, 5- and 50-µmol/L H$_2$O$_2$ treatment was able to increase phospho-Akt levels (Fig. 6D), while catalase expression abrogated Akt phosphorylation (Supplementary Fig. S8), suggesting that this may be an important redox-dependent signaling pathway in OCCC.

It was noted that ES-2 control cells were not able to tolerate long-term exposure to low-dose H$_2$O$_2$ during migration assays (Fig. 6C). To examine this further, cell viability was assessed in response to H$_2$O$_2$. A significant reduction in cell survival in response to H$_2$O$_2$ was observed in the control group compared with cells with decreased Sod2 expression (Fig. 7A). This suggests that a higher intracellular steady-state H$_2$O$_2$ milieu in OCCC predisposes cells to enhanced killing by additional exposure to low-level exogenous H$_2$O$_2$. The above data imply that high Sod2 expression provides several advantages to OCCC by protecting mitochondrial function through scavenging of O$_2^-$ and driving H$_2$O$_2$-dependent migration. While these attributes are advantageous for OCCC survival and metastatic progression, an enhanced intracellular steady-state H$_2$O$_2$ level presents a double-edged sword, as these cells are consequentially more susceptible to H$_2$O$_2$ toxicity (Fig. 7B).

**Discussion**

Although the five different EOC histologic subtypes share the same primary tumor location on the ovaries, it is now evident that these are distinct diseases with vastly different tissue origins and genetic and epigenetic profiles (2, 11). In the present study, we show that Sod2 is highly expressed in OCCC compared with other EOC histologic subtypes and that this mitochondrial antioxidant plays a significant role in OCCC tumorigenicity and metastasis.

Intracellular ROS are maintained within a narrow range tightly regulated by the balance of the rate of ROS production and ROS scavenging/detoxifying by antioxidant enzymes. This balance is often disrupted in the context of cancer, due to high ROS production as a consequence of changes in metabolism or the tumor environment (e.g., hypoxia) and the resulting changes in antioxidant expression. Because the mitochondrial respiratory chain is the major site of O$_2^-$ generation within cells, Sod2 plays an important role in maintaining cellular ROS balance. On the basis of the above findings, Sod2 appears to play a dual role in enhancing OCCC tumorigenicity, first, by protecting cells from mitochondrial O$_2^-$ damage, and second, by shifting the steady-state ROS balance toward H$_2$O$_2$.

We recently demonstrated that a distinguishing feature of OCCC is their unique metabolic phenotype. Compared with serous adenocarcinoma cells, OCCC cell lines were significantly more energetic, displaying both very high levels of mitochondrial oxidative phosphorylation and glycolytic flux (13). Our data suggest that Sod2 is intricately involved in maintaining this high rate of oxygen consumption, potentially by preserving mitochondrial function as a consequence of O$_2^-$ scavenging (Fig. 3). By preventing mitochondrial electron transport chain complex damage mediated by O$_2^-$ or secondary products, Sod2 likely supports the high rate of OCCC proliferation, clonogenicity, and tumor growth. The results of the present study suggest that inhibiting mitochondrial antioxidant defenses may provide an alternative strategy to therapeutically target OCCC.

In addition to scavenging O$_2^-$ and maintaining mitochondrial health for optimal cell proliferation, we believe that Sod2 has another role in promoting the aggressiveness of OCCC, by shifting steady-state H$_2$O$_2$ levels and driving prometastatic behavior (Figs. 4–6). It has been previously shown that high expression of Sod2 is associated with metastatic progression (8, 10, 17, 20–23) and that dependence of cancer cell migration is related to cellular H$_2$O$_2$ production (9, 19, 24, 25). For example, we have

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**Figure 7.** Exogenous H$_2$O$_2$ treatment significantly decreases viability of cells with higher steady-state H$_2$O$_2$ levels. A, ES-2 scramble control cells show significantly lower viability than shSod2 ES-2 cells using crystal violet assays, in response to increasing concentrations of H$_2$O$_2$ for 72 hours. Each data point represents an average of 6 replicates ± SEM. Tukey posttest: *p < 0.05. B, proposed mechanisms of Sod2-mediated OCCC tumorigenesis and metastasis. OCCC has high Sod2 expression, which provides efficient superoxide scavenging and maintenance of high mitochondrial function to drive increased cell proliferation. In addition, Sod2 shifts the intracellular ROS balance from O$_2^-$ to H$_2$O$_2$, which drives tumor cell migration and metastasis. This concomitantly enhances the intracellular H$_2$O$_2$ threshold, enabling more rapid killing by exogenous H$_2$O$_2$.**

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shown that steady-state increases in H₂O₂ can lead to induction of the FAK pathway and migration of metastatic bladder cancer cells and cells with enforced Sod2 expression (9, 19). This effect was mediated by oxidation-dependent inhibition of the phosphatase PTPN12, leading to enhanced phosphorylation of p130cas and Rac1 activation. In addition, work from the Melendez group has shown that Sod2 expression significantly contributes to the expression of the matrix-degrading enzyme MMP-1 in an H₂O₂-dependent manner (21) and that the Sod2/H₂O₂-dependent inhibition of the dual-lipid protein tyrosine phosphatase PTEN enhances Akt/GSK3β/VEGF-dependent angiogenesis (18), both processes contributing significantly to metastasis. Our present data suggest that Sod2 may similarly contribute to metastatic progression of OCCC by activating Akt signaling (Figs. 5 and 6). In addition to its prosurvival function, Akt has been shown to influence metastasis and cell migration by regulating cytoskeletal rearrangement, prometastatic cell signaling, and gene transcription (26). These results are of specific importance to OCCC, which, unlike other ovarian cancer histologic subtypes, has been characterized by high-frequency Akt pathway activation. Seventy percent of early- and 68% of late-stage OCCC cases have been shown to display phosho-Akt (S473) staining (27). About 38% of OCCC cases show PTEN loss (28) and 40% of cases PI3K activating mutations (29). Our data imply that Sod2-dependent Akt phosphorylation may also contribute to high activation of Akt signaling in OCCC. Because Akt phosphorylation was highly susceptible to H₂O₂ treatment, it suggests that this signaling pathway is redox-regulated in OCCC, with a plausible mechanism for this being the oxidation of PTEN (18).

Although an increase in Sod2 should theoretically not result in higher levels of H₂O₂ production based on the enzyme’s kinetic properties (30), a number of studies have demonstrated increases in H₂O₂ levels that correlate with Sod2 expression (18, 31–33). While the reason for this observation in OCCC has not been investigated, there are plausible explanations for this increase in steady-state H₂O₂ as a consequence of Sod expression. These explanations primarily relate to changes in the reaction rates within the mitochondrial electron transport chain (ETC). For example, it has been proposed that Sod2 in the mitochondria may alter the flux of O₂·– from some quinone/semiquinone/hydroquinone triads, such as coenzyme Q, thereby driving the reaction into the direction of O₂·– production, potentially leading to enhanced localized dissmutation to H₂O₂ by Sod2 (31, 34). Alternatively, inhibition of cytochrome c oxidase by nitric oxide, arising as a consequence of Sod2 expression, may influence the reduction state of the ETC and drive O₂·– and H₂O₂ production (35). Our observation that Sod2 knockdown also decreases H₂O₂ levels suggests that Sod2 is involved in regulating H₂O₂ balance within cells, and this may contribute to H₂O₂-mediated redox signaling.

While Sod2 appears to contribute to H₂O₂-mediated metastatic progression, an enhanced steady-state H₂O₂ milieu may also present a disadvantage to OCCC cells. Our data suggest that cells with high Sod2 levels and concomitant increases in intracellular H₂O₂ are more susceptible to exogenous sources of redox stress (Fig. 7A). This likely puts cells closer to the cytotoxic threshold of H₂O₂, which is reached once cells are further challenged by exogenous ROS. Interestingly, OCCC cells do not appear to have enhanced expression of catalase to provide additional scavenging of excess H₂O₂ (Fig. 6A). While sublethal levels of H₂O₂ have been shown to contribute to redox signaling, high levels of H₂O₂ can elicit tumor cell death by a number of pathways, including apoptosis, protein/DNA damage, and mitochondrial dysfunction (36–38). Furthermore, it was recently reported that H₂O₂ exposure of tumor cells with enforced Sod2 expression can result in Sod2 peroxidase activity, leading to mitochondrial damage and dysfunction (39). An increased H₂O₂ steady-state has been observed in a number of cancer cells (17, 40–42) and lends credence to the idea that this higher H₂O₂ threshold may be exploited therapeutically. In this regard, the use of high-dose ascorbic acid, which is oxidized within tumor cells to produce H₂O₂, has recently been revisited for use in cancer treatment (43–45) and has shown promise in early clinical trials in advanced-stage cancers (46, 47). Ascorbate and concomitant H₂O₂-mediated DNA damage and apoptosis were shown to enhance ovarian cancer cell death and increase chemosensitivity (48). While that study was not focused on OCCC, this type of treatment may be of particular benefit to this histologic subtype given the high expression of Sod2. It is important to highlight that cancer cells with enhanced Sod2 expression may respond differently to ROS-producing agents, depending on both the type and cellular location of the ROS/reactive nitrogen species generated. For instance, Sod2 may enhance scavenging of O₂·– and therefore provide chemoresistance benefits to the tumor cells in response to these ROS. Conversely, while an increase in steady-state H₂O₂ facilitates redox signaling beneficial to the cancer cells, this higher threshold may facilitate H₂O₂-mediated OCCC cell death in response to further insult by exogenous sources of H₂O₂. Understanding the complex interaction of antioxidants and ROS in OCCC is therefore of importance and may provide novel therapeutic avenues to pursue for this histologic subtype of ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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