Quiescin Sulphydryl Oxidase 1 Promotes Invasion of Pancreatic Tumor Cells Mediated by Matrix Metalloproteinases

Benjamin A. Katchman1, Kwasi Antwi1, Galen Hostetter2, Michael J. Demeure2, April Watanabe2, G. Anton Decker3, Laurence J. Miller3, Daniel D. Von Hoff2, and Douglas F. Lake1

Abstract

Quiescin sulphydryl oxidase 1 (QSOX1) oxidizes sulphydryl groups to form disulfide bonds in proteins. We previously mapped a peptide in plasma from pancreatic ductal adenocarcinoma (PDA) patients back to an overexpressed QSOX1 parent protein. In addition to overexpression in pancreatic cancer cell lines, 29 of 37 patients diagnosed with PDA expressed QSOX1 protein in tumor cells, but QSOX1 was not detected in normal adjacent tissues or in a transformed, but nontumorigenic cell line. To begin to evaluate the advantage QSOX1 might provide to tumors, we suppressed QSOX1 protein expression using short hairpin (sh) RNA in two pancreatic cancer cell lines. Growth, cell cycle, apoptosis, invasion, and matrix metalloproteinase (MMP) activity were evaluated. QSOX1 shRNA suppressed both short and long isoforms of the protein, showing a significant effect on cell growth, cell cycle, and apoptosis. However, QSOX1 shRNA dramatically inhibited the abilities of BxPC-3 and Panc-1 pancreatic tumor cells to invade through Matrigel in a modified Boyden chamber assay. Mechanistically, gelatin zymography indicated that QSOX1 plays an important role in activation of MMP-2 and MMP-9. Taken together, our results suggest that the mechanism of QSOX1-mediated tumor cell invasion is by activation of MMP-2 and MMP-9. Mol Cancer Res; 9(12): 1621–31. ©2011 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is a disease that carries a poor prognosis. It is often detected in stage III resulting in an unresectable tumor at the time of diagnosis. However, even if pancreatic cancer is surgically resected in stage I or II, it may recur at a metastatic site (1, 2). Currently, patients diagnosed with PDA have less than a 5% chance of surviving past 5 years (3). Through proteomic analysis of pancreatic cancer patient plasma, we discovered a peptide form, quiescin sulphydryl oxidase 1 (QSOX1), that maps back to the C-terminus of the long isoform of QSOX1 (QSOX1-L; ref. 4). Subsequently, we found that QSOX1 is overexpressed in tumor tissue from pancreatic cancer patients but not adjacent normal tissue (Fig. 1B and C). These findings led us to hypothesize that overexpression of QSOX1 might be functionally important for tumor cells, prompting further exploration of the role that QSOX1 might play in pancreatic cancer.

QSOX1 belongs to the family of FAD-dependent sulphydryl oxidases that are expressed in all eukaryotes sequenced to date. As eloquently shown by the Thorpe and Fass laboratories, the primary enzymatic function of QSOX1 is oxidation of sulphydryl groups during protein folding to generate disulfide bonds in proteins, ultimately reducing oxygen to hydrogen peroxide (5–7). QSOX1 has been reported to be localized to the Golgi apparatus and endoplasmic reticulum (ER) in human embryonic fibroblasts, where it works with protein disulfide isomerase (PDI) to help fold nascent proteins in the cell (8, 9).

In the human genome, QSOX1 is located on chromosome 1q24 and alternative splicing in exon 12 generates a long (QSOX1-L) and short (QSOX1-S) transcript (Fig. 1A; ref. 10). Both, QSOX1-S and QSOX1-L have identical functional domain organization from the amino terminus as follows: two thioredoxin-like domains (Trx1 and 2), a helix-rich region (HRR), and an Env/ALR FAD-binding domain (5, 11). QSOX1-L contains a predicted transmembrane domain that is not present in QSOX1-S due to alternative splicing (Fig. 1A; ref. 12). QSOX1 was originally discovered in quiescent human lung fibroblasts and was hypothesized to aid in the transition from G0 to S phase of the cell cycle, a balance often altered in cancer cells (13, 14). Since the initial discovery of QSOX1, the majority of research to date has focused on detailing the sulphydryl oxidase activity for disulfide bond formation in proteins.
Thorpe and colleagues revealed the ability of QSOX1 to efficiently generate disulfide bonds into proteins during folding at rate of 1,000 per minute with a $K_M$ of 150 $\mu$mol/L per thiol (7). QSOX1 seems to play a significant role in redox regulation within the cell, although the in vivo biological substrates are undefined, as well as the functional significance associated with each splice variant.

A recent publication by Morel and colleagues highlighted the role of QSOX1 in protecting MCF7 breast tumor cells from oxidative stress–induced apoptosis (15). They found that expression of QSOX1 directly correlates with an increase in cell survival after treatment with H$_2$O$_2$ (15). These findings may suggest that increased QSOX1 expression in tumor cells allows them to actively evade cellular apoptotic mechanisms mediated by reactive oxygen species.

Further evidence supporting an active role for QSOX1 in prostate tumor cells was recently reported by Song and colleagues (16). They found that loss of NKX3.1 expression correlates with an increase in QSOX1 expression in prostate tumors. NKX3.1 is a homeobox transcription factor and a known tumor suppressor that is exclusively expressed in luminal epithelial cells of the prostate. QSOX1 was shown to be highly expressed in early stages of prostatic neoplasia and throughout prostate cancer progression, but it was not present in normal prostate (16, 17). This finding mirrors our initial discovery of QSOX1 overexpression in pancreatic cancer (4). NKX3.1 expression is decreased or absent in early stages of prostate tumor development and absent in up to 80% of metastatic prostate tumors (18). Because loss of NKX3.1 results in overexpression of QSOX1, the oxidative environment that occurs because of QSOX1 activity may play a role in early tumorigenesis (16).

In this study, we have begun to analyze the role of QSOX1 in pancreatic tumors using cell lines BxPC3 and Panc-1. We knocked down QSOX1-S and QSOX1-L protein expression using short hairpin RNAs (shRNA) in an attempt to reveal how pancreatic cancer cells might be affected. We assessed cell growth, cell cycle, apoptosis, invasion, and matrix metalloproteinase (MMP) activity. QSOX1 knockdowns affected tumor cell proliferation, cell cycle, and apoptosis. We observed a dramatic decrease in tumor cell invasion when QSOX1 expression was suppressed. Further investigation into the mechanism of invasion revealed that QSOX1 is at least partially responsible for MMP-2 and MMP-9 activity. This is the first article showing a role for QSOX1 in invasion and metastasis.

Figure 1. QSOX1 is highly expressed in tumor cell lines but is not expressed in adjacent normal cells. Previously, our laboratory discovered a short peptide, NEQEQPLGQWHLS, in patient plasma through liquid chromatography/tandem mass spectrometry. We were able to map this short, secreted peptide back to an understudied parent protein, QSOX1-L. A, diagram showing the two splice variants of QSOX1, QSOX1-Short (S) and -Long (L), both contains a thioredoxin 1 (Trx1) and ERV/ALR functional domains as well as structural thioredoxin 2 (Trx2) and HRR. QSOX1-L contains a predicted transmembrane (TM) domain. The peptide NEQEQPLGQWHLS, maps back to QSOX1-L, and found to be secreted in pancreatic cancer patients but not in normal samples. The commercially available antibody recognizes the first 329 amino acids of both QSOX1-S and -L. B, Immunohistochemistry of normal (left) and tumor (right) pancreatic tissue sections that have been stained with the anti-QSOX1 showing tumor specific staining in pancreatic ducts but not in adjacent nontumor cells. C, Western blot analysis of patient tumor as well as adjacent normal tissue indicates that QSOX1-S is the dominant splice variant expressed. D, Western blot showing QSOX1 expression in transformed normal pancreatic cells (HPDE6) and human pancreatic adenocarcinoma cells (Panc-1, CFPac-1, BxPC3, and Capan1) shows that our in vitro system mimics that of the in vivo QSOX1 expression as shown above using IHC. Western blots have been cropped and full Western blots can be viewed in Supplementary Fig. S3.

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Material and Methods

Cell culture
Pancreatic adenocarcinoma BxPC3, PANC-1, CFPac-1, and Capan1 cancer cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS (Gibco). Immortal human nonmalignant pancreatic duct epithelial cells (HPDE6) were cultured in Clontech KGM-2 karotinoocyte media (Gibco; ref. 19). All cell lines were grown at 37°C with 5% CO2. Cell lines are tested monthly for Mycoplasma contamination using Venor GeM Mycoplasma Detection Kit, PCR based from Sigma.

Immunohistochemistry
Immunohistochemistry (IHC) on patients who underwent surgical resection was done in the exact manner as previously described in Kwasi and colleagues (4).

Generation of shRNA and lentiviruses production
Three different shRNA for QSOX1 were obtained through DNASU (http://dnasu.asu.edu; ref. 20) already in the lentiviral pLKO.1-puromycin selection vector. QSOX1 sh742, 5'-CCGGGCAATAAGTGTTGGAAGAAG TTTCGAGAACATTTTCTCACCATTTGCTTTTTTGTGTA-3' (sense), QSOX1 sh528, 5'-CCGGCAATAAGAGAAGGCTTT-3' (sense), QSOX1 sh616, 5'-TCTAGCCACACAGGGTGGCATGTT-3' (sense) and shScramble with target sequence 5'-TCCGTGGTTGGAACAGCGCTTTT-3' was obtained from Josh LaBaer’s laboratory at Arizona State University. The target sequence is in italics and each vector contains the same supporting sequence surrounding the target sequence. Lentiviruses containing sh742, sh528, sh616, and shScramble were produced using 293T cells. 293T cells were transfected with 2,500 ng shRNA maxi-prepped viral pLKO.1-puromycin selection vector. QSOX1 sh742, sh528, sh616, and Capan1 cancer cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS (Gibco). (sense), QSOX1 sh616, 5'-TCTAGCCACACAGGGTGGCATGTT-3' (sense) and shScramble with target sequence 5'-TCCGTGGTTGGAACAGCGCTTTT-3' was obtained from Josh LaBaer’s laboratory at Arizona State University. The target sequence is in italics and each vector contains the same supporting sequence surrounding the target sequence. Lentiviruses containing sh742, sh528, sh616, and shScramble were produced using 293T cells. 293T cells were seeded at 1.5 × 10^6 cells per well in 2 mL media lacking antibiotics, using a 6-well plate format and incubated at 37°C, 5% CO2 for 24 hours. The following day, the 293T cells were transfected with 2,500 ng shRNA maxi-prepped plasmid DNA (Sigma GeneElute HP Plasmid Maxiprep Kit), 500 ng VSVG, 2,500 ng d8.91 (gag-pol) in LT1 transfection reagent from Mirus Bio and centrifuged at 1,000 × g for 30 minutes and incubated as 37°C, 5% CO2 for 24 hours in media lacking antibiotics. The next morning media containing lentivirus was collected and replaced with complete media. Supernatants (2.5 mL) from transfected 293T cells producing each sh lentivirus were collected every 24 hours for a total of 72 hours, combined, and stored at −20°C.

Generation of shQSOX1-transduced tumor cell lines
Stable transduction of sh742, sh528, sh616, and shScramble into BxPC-3 and Panc-1 cell lines was done by first seeding the cells at 8 × 10^3 cells per well in a 6-well plate and incubating overnight. The next day, the cells were transduced by adding 8 μg/mL polybrene (Millipore) and 200 μL sh742, sh528, sh616, and shScramble lentivirus media from 293T cells to each well. The cells were spun at 1,000 rpm for 30 minutes and then incubated for 24 hours. The following day, fresh DMEM with 10% FBS was added, containing 1 μg/mL puromycin (Sigma); to select for the transduced cells, QSOX1 knockdown was measured by Western blot.

SDS-PAGE-Western blotting
Western blotting was done using cell lysates from HPDE6, BxPC3, Panc-1, Capan1, and CFPac1 cells, as well as patient 1010 and 1016 tumor and adjacent normal enzymatic supernatant. Cell lysates were generated by harvesting 2.5 × 10^6 cells by centrifugation, followed by lysis using radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% Triton X-100) with 1× SigmaFAST Protease Inhibitor Cocktail Tablet, EDTA Free. Protein in the cell lysate was measured using the micro BCA protein assay kit (Thermo Scientific). All samples were then normalized to 2 μg/mL (20 μg total protein per lane). Samples were run on 10% SDS-polyacrylamide gels, then transferred onto Immob-Blot polyvinylidene difluoride Membranes (Bio-Rad). Rabbit polyclonal anti-QSOX1 (ProteinTech), rabbit polyclonal anti-Bactin and anti-alpha-tubulin (Cell Signaling), and rabbit polyclonal anti–MMP-2 and MMP-9 (Sigma) antibody was diluted by 1:1,000, 1:1,000, and 1:500, respectively, in 0.1% bovine serum albumin in 1× TBS+0.01% Tween-20 and incubated overnight. Goat anti-rabbit IgG-alkaline phosphatase or horseradish peroxidase (HRP) secondary antibody was used at a 1:5,000 dilution and incubated with the blot for 1 hour followed by washing. BCIP/NBT substrate (Pierce Chemical) was added and the blot was developed at room temperature (RT) for approximately 1 hour, in samples incubated in alkaline phosphatase secondary antibody. For samples incubated in goat anti-rabbit HRP secondary, the blots were developed using Novex ECL Chemiluminescent Substrate Reagent Kit. Quantification of band intensity was measured using ImageJ and is presented as percent change from the scrambled shRNA control. Full gel images are available in the Supplementary Material. All gel images were annotated and processed using Photoshop software.

MTT assay
Cells were seeded at 3 × 10^5 cell per well in a 96-well plate in triplicate and incubated at 37°C, 5% CO2 over the course of 5 days. The MTT assay was done on days 1, 2, and 5 according to the manufacturer’s instructions (Invitrogen-Molecular Probes, Vybrant MTT Cell Proliferation Assay Kit). Results are presented as mean ± SD. Student 2-tailed t test was done to determine significance.

Annexin V/propidium iodide apoptosis analysis
Apoptosis analysis was done according to the manufacturer’s instructions [fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I; BD Pharmingen]. Briefly, cells were seeded at equal densities in a 25-cm² flask until they reached 60% to 80% confluence. The cells were then washed with cold PBS, counted, and normalized to 1 × 10^5 cell/mL in 1× Annexin V binding buffer. Next, 1 × 10^5 cells were then transferred to a separate tube and 5 μL of FITC Annexin V and 5 μL of propidium iodide were added
to each sample. The samples were gently vortexed and incubated for 15 minutes at RT in the dark. Finally, 400 μL of 1× binding buffer was added to each sample and the samples were analyzed by flow cytometry (Becton Dickinson FACSCalibur FlowCytometer) with 1 hour. Each sample was done in triplicate.

RNA isolation and cDNA synthesis
Total RNA isolation was done according to the manufacturer’s instructions for animal cells using spin technology (RNeasy Mini Kit; Qiagen). After RNA was isolated from each sample, it was reverse transcribed with qScript cDNA Synthesis Kit, Quanta Biosciences according to the manufacturer’s instructions.

Quantitative real-time PCR
The relative level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), QSOX1-L, QSOX1-S, MMP-2, and MMP-9 were analyzed in each sample by quantitative real-time PCR (qPCR). Each cDNA sample was normalized to the expression of PerfeCTa SYBR Green Fast Mix, ROX to a final concentration of each primer and 1× final concentration of PerfeCta SYBR Green Fast Mix, ROX from Quanta Biosciences on a ABI7900HT thermocycler, Applied Biosystems Inc. Reactions were done in triplicate.

MMP-9 were analyzed in each sample by quantitative real-time PCR (qPCR). Each cDNA sample was normalized to the expression of PerfeCTa SYBR Green Fast Mix, ROX to a final concentration of each primer and 1× final concentration of PerfeCta SYBR Green Fast Mix, ROX from Quanta Biosciences on a ABI7900HT thermocycler, Applied Biosystems Inc. Reaction Protocol: Initial denaturation at 95°C for 3 minutes; PCR cycling (40 cycles) i, 95°C, 30 seconds. ii, 55°C, 30 seconds. iii, 72°C, 1 minutes; melt curve (dissociation stage). The primer sequences for the genes analyzed are as follows: GAPDH forward 5'-GGCGCTCCAAGGAGTAAAGCC; GAPDH Reverse 5'-AGGGTGCTCTACATGGCAACTG; QSOX1-L forward 5'-TGTGCTAGCCACACAGGCTTCAAT; QSOX1-S reverse 5'-TGTGCGACGCCAGAAACAATTTCA; QSOX1-L forward 5'-TGTGCTAGCCACACAGGCTTCAAT; QSOX1-S reverse 5'-TGTGCGACGCCAGAAACAATTTCA; Each reaction was done in triplicate, with the data representing the averages of one experiment.

In the shRNA experiment, expression of MMPs was normalized to the nontargeted GAPDH to determine ΔCq. ΔCq replicates were then exponentially transformed to the ΔΔCq expression after which they were averaged ± SD. The average was then normalized to the expression of the shScramble control to obtain the ΔΔCq expression. Significance was determined using the Student’s 2-tailed t test.

Matrigel invasion assay
Invasion assays were done using BD BioCoat BD Matrigel Invasion chambers with 8.0-μm pore size polyethylene terephthalate membrane inserts in 24-well format. The assay was done according to the manufacturer’s instructions (BD Bioscience). A total of 4 × 10^5 cells per well were seeded into the inner Matrigel chamber in serum-free DMEM. The outer chamber contained 10% FBS in DMEM. BxPC3 and Panc-1 cells were incubated for 24 hours at 37°C, 5% CO2. Cells that invaded through the Matrigel and migrated through the pores onto the bottom of the insert were fixed in 100% methanol and then stained in hematoxylin (Invitrogen). The total number of invading cells were determined by counting the cells on the underside of the insert from 3 wells (6 fields per insert) at 10×, 20×, and 40× magnification, and the extent of invasion was expressed as the mean ± SD. Significance was determined using the Student’s 2-tailed t test. Results presented are from 1 of 3 independent experiments.

Gelatin zymography
The identification of MMP was done using gelatin zymography. Zymography experiments were done as follows. Untreated BxPC3 and Panc-1 cells as well as transduced cells were seeded at 5 × 10^5 cells per well (12-well plates) in DMEM with 10% FBS. The next day, cells were then washed with 1×PBS and the media was changed to serum-free DMEM and incubated for 24 hours before being collected and protein concentrations determined using a BCA assay. Gelatin zymography was done with a 10% polyacrylamide gel containing gelatin solution in place of water (0.8 mg/mL gelatin, 0.15 mol/L Tris pH 8.8, 30% acrylamide-bis, 50% glycerol, 10% SDS, 10% APS, and TEMED; ref. 21). A volume of equal concentrations of serum-free conditioned media were loaded under nondenaturing conditions into the 10% polyacrylamide–gelatin gel to separate proteins secreted by the tumor cells and to detect the presence of gelatin degrading MMPs. Electrophoresis was done at a constant voltage of 150 V for 60 minutes. Gels were washed in renaturing buffer (25% Triton X-100 in water) for 30 minutes at RT with gentle shaking. The gels were then equilibrated in developing buffer (50 mmol/L Tris-base, 6.3 g/L Tris-HCl, 0.2 mol/L NaCl, 5 mmol/L CaCl2, and 0.02% Triton X-100) for 30 minutes at RT with gentle shaking. Fresh developing buffer was then added to the gels and they were incubated overnight at 37°C. The gels were then stained with SimplyBlue Safe Stain (Invitrogen) for 20 minutes at RT, then destained overnight in ddH2O at RT. The presence of MMP was detected by the lack of staining indicating digestion of gelatin. The negative control was done by adding 50 mmol/L EDTA to both the renaturing buffer and the developing buffer to block the MMP activation (Supplementary data S3). Quantification of band intensity was measured using ImageJ and is presented as percent change from the scrambled shRNA control.

Results
Detection of QSOX1 by immunohistochemistry and Western blot
To begin to determine the frequency of expression of QSOX1 in human PDA, QSOX1 expression was assessed in 4 different pancreatic tumor cell lines, an immortal non-tumorigenic cell line, HPDE6, 37 tumor tissue sections from...
patients with PDA, and tumor and adjacent normal tissue from 2 patients, 1016 and 1010 (Fig. 1B–D). Twenty-nine of 37 tumor tissues were positive for QSOX1 expression, suggesting it is a commonly overexpressed protein. To determine which splice variant was more prevalent in our IHC images, we analyzed tumor as well as adjacent normal tissue from 2 patients by Western blot (Fig. 1C). Our results revealed that QSOX1-S is the dominant splice variant expressed, also corroborating our IHC results that revealed an increase in QSOX1 expression in tumor samples. Although our adjacent normal samples indicate a high level of QSOX1 expression, it is hard to determine whether there was any tumor tissue contaminating our normal sample, which would account for the increase in QSOX1 expression. Western blotting analysis shows that 4 pancreatic tumor cell lines, BxPC3, Panc-1, Capan1, and CFPac-1 strongly express QSOX1-S and weakly express the longer splice variant, QSOX1-L. HPDE6, an immortal, nontumorigenic pancreas epithelial cell line, shows weak expression of QSOX1-S and no expression of QSOX1-L (Fig. 1D).

The results of this experiment begin to provide some information about the frequency and distribution of QSOX1 expression. First, QSOX1 seems to be a commonly overexpressed protein in PDA (Fig. 1B and C). Second, QSOX1 protein expression in adjacent normal 1016, 1010, and HPDE6, a nontumorigenic pancreatic duct cell line, is much weaker than it is in the patient tumor samples and 4 malignant pancreatic tumor cell lines. This may suggest that QSOX1 provides some advantage to malignant cells that nonmalignant cells do not require.

QSOX1 promotes tumor cell proliferation

To examine the advantage that QSOX1 provides to tumor cells, we inhibited QSOX1 expression in BxPC3 and Panc-1 cells using 3 shRNA constructs: sh742, sh528, and sh616. shScrambled was generously provided by Dr. Joshua LaBaer. Lentiviruses containing each shRNA were generated as described in "Methods." BxPC3 and Panc-1 cells were transduced with each sh-lentivirus (shQSOX1) to evaluate the effects of QSOX1 knockdown on tumor cell growth. To show that the shQSOX1 are active in both cell lines, Fig. 2A and B shows reduced protein expression of both isoforms of QSOX1 in BxPC3 and Panc-1 cells assayed on day 1, 2, and 5. Data represent averages ± SD. Significance *<0.05; **<0.01.

Figure 2. Reduced expression of QSOX1 in BxPC3 and Panc-1 cells leads to a significant decrease in cell growth. To determine the phenotype presented due to the expression of QSOX1 in tumor cells, we employed shRNA specific to QSOX1 to reduce the expression of QSOX1 in A, BxPC3 (percent decrease in sh742–56%; sh528–40%; sh616–28%) and B, Panc-1 (percent decrease in sh742–64%; sh528–46%; sh616–18%) cells and further evaluated cell growth, cell cycle, apoptosis, and invasion/metastasis. Western blots have been cropped and full images can be viewed in Supplementary Fig. S3. C, MTT assay on shRNA treated BxPC3 and Panc-1 cells assayed on day 1, 2, and 5. Data represent averages ± SD. Significance *<0.05; **<0.01; ***<0.001.

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growth; by day 5 BxPC3 sh742, sh528, and sh616 showed a 65%, 60%, and 37% decrease, whereas in Panc-1 sh742, sh528, and sh616, there was an 84%, 88%, and 61% decrease. Live cell counts using trypan blue confirmed the MTT assay (Supplementary Fig. S1).

Cell-cycle and apoptosis analysis

Previous work has correlated QSOX1 expression with the quiescent stage, G0, of the cell cycle (10), leading us to hypothesize whether the shQSOX1-mediated decrease in cell proliferation was the result of abnormal regulation of the cell cycle or an increase in apoptosis. To address this hypothesis, propidium iodide (PI) was used in flow cytometry to evaluate the effects of shQSOX1 on cell cycle. Our results indicate that suppression of QSOX1 expression did modulate cell cycle in both BxPC3 and Panc-1 compared with our untreated and scrambled control (Supplementary Fig. S2). The results show that the reduced expression of QSOX1 on cell cycle could be cell dependent. BxPC3 showed an increase in G1 and a significant decrease in S, whereas Panc-1 cells showed a significant decrease in G1 but no changes in S (Supplementary Fig. S2).

We further evaluated whether the decrease in cellular proliferation mediated by shQSOX1 was due to an increase in apoptotic cell death. To assess apoptosis, BxPC3 and Panc-1 cells transduced with shScramble, sh742, sh528, and sh616 were stained with Annexin-V and PI. Compared with untreated and shScramble, a consistent increase of 2% to 8% in early and late apoptosis (Annexin-V single and double positive) was observed for each of the shQSOX1 constructs in BxPC3 and Panc-1 cells. Our results clearly show that knockdown of QSOX1 expression in tumor cells leads to a dramatic decrease in the number of pancreatic tumor cells that degrade Matrigel and migrate through the insert into nutrient-rich media.

Role of QSOX1 in tumor cell invasion

For a tumor cell to invade other tissues as part of the metastatic process, the cell must first degrade basement membrane components such as laminin, collagen, and fibronectin before they can migrate into the bloodstream and reestablish itself in a distant organ (3). To evaluate whether overexpression of QSOX1 in BxPC3 and/or Panc-1 cells plays a role in metastasis, we carried out invasion assays over an 18-hour period. Untreated, shScramble, sh742, sh528, and sh616-transduced cells were plated in serum-free medium on Matrigel-coated, 8-μm pore inserts. Inserts were placed into wells containing 10% FBS in DMEM. After 18 hours of incubation, tumor cells that had degraded Matrigel and migrated through 8-μm pores onto the underside of the insert were counted (Fig. 4A and B). Our results clearly show that knockdown of QSOX1 expression in tumor cells leads to a dramatic decrease in the number of pancreatic tumor cells that degrade Matrigel and migrate through the insert into nutrient-rich media.

Mechanism of invasion

Because knockdown of QSOX1 protein expression in pancreatic tumor cells lines decreases invasion through Matrigel, it was important to determine the mechanism of inhibition of the invasive process. MMP-2 and MMP-9 are key contributors of invasion and metastasis in pancreatic cancer (2). Both, pro-MMP-2 and MMP-9 mRNA and protein levels are elevated in pancreatic tumors, and activated MMP-2 (a-MMP-2) seems to be key contributors of metastasis in PDA (2, 22). Because QSOX1 has been suggested to be secreted into the extracellular matrix, where MMPs are thought to be activated, we hypothesized that QSOX1 might help activate MMP-2 and MMP-9 proteins. Untreated BxPC3 and Panc-1 cells, as well as transduced shScramble, sh742, sh528, and sh616 were incubated for 18 to 24 hours in serum-free media after which supernatants were collected and subjected to gelatin-SDS-PAGE. Gelatin zymography
Figure 4. Reduced expression of QSOX1 in BxPC3 and Panc-1 cells leads to a significant decrease in cellular invasion. A, untreated BxPC3 and B, untreated Panc-1 cells were treated with scramble, sh742, sh528, and sh616 shRNAs specific for QSOX1 and seeded in the top chamber of Matrigel invasion wells and allowed to incubate for 18 hours. Representative 10×, 20×, and 40× images are presented. In the BxPC3 sh742, sh528- and sh616-treated cells, there was an 84%, 84%, and 79% decrease in cells that were able to break down the basement membrane components of the Matrigel and invade to the underside of the membrane, respectively; whereas in Panc-1 sh742, sh528, and sh616 cells, there was a 76%, 76%, and 63% decrease in cells that were able to degrade the Matrigel and invade through the membrane. Graphs represent average ± SD (BxPC3 n = 6; PANC-1 n = 3), significance *, P < 0.05; **, P < 0.005.
was done to determine whether QSOX1 plays a role in secretion and/or activation of MMPs.

Our first observation from this experiment is that BxPC3 and Panc-1 have very different zymographic profiles. BxPC3 supernatants contain MMP-9 homodimer (130 kDa), a large amount of proteolytically active pro-MMP-9 (92 kDa) with lesser concentrations of pro-MMP-2 (72 kDa) and a-MMP-2 (66 kDa). Panc-1 supernatants contain less prominent MMP-9 homodimer, pro-MMP-9 (92 kDa), and a large amount of proteolytically active pro-MMP-2 (72 kDa), unlike BxPC3 cells.

Supernatants from BxPC3 cells transduced with sh742, sh528, and sh616 showed a 65%, 47%, and 10% decrease, respectively, in pro-MMP-9 compared with shScramble (Fig. 5A). Supernatants from Panc-1 cells transduced with sh742, sh528, and sh616 showed a 70%, 56%, and 15% decrease, respectively, in pro-MMP-2. Sh616 did not knockdown QSOX1 as effectively as sh528 and sh742, and this is reflected in the cell growth, invasion, and MMP activity. Thus, decreases in the proteolytic activity of MMP-2 and MMP-9, using gelatin as a substrate, provide a mechanism for the shQSOX1-mediated suppression of invasion through Matrigel.

To confirm our gelatin zymography results, we used Western blot analysis of BxPC3 and Panc-1 serum-free conditioned media to probe for MMP-2 and MMP-9 (Fig. 5C). Although our results indicate a slight decrease in MMP-2 and MMP-9 (between 1% and 10% decrease using densitometric analysis) in BxPC3 and Panc-1 shQSOX1-treated cells, it is nowhere near the level shown using gelatin zymography. This could be explained as a difference between a functional assay, gelatin zymography, and a purely quantitative assay, such as Western blot.

To extend our hypothesis that QSOX1 is influencing MMPs posttranslationally, we conducted qRT-PCR on MMP-2 and MMP-9 comparing the transcripts from shQSOX1-transduced cell lines with shScrambled. Figure 5 shows that MMP-2 and MMP-9 RNA increased in the shQSOX1-transduced cells compared with control cells. This result adds confidence to our hypothesis that QSOX1 does not transcriptionally suppress MMP production; rather, it posttranslationally activates MMPs. It also diminishes the possibility that shQSOX1 RNAs are suppressing MMP transcription due to off-target effects.

**Discussion**

The mortality rate for patients diagnosed with pancreatic cancer has remained stagnant for the last 5 decades, despite advanced surgical procedures and improvements in chemotherapeutics (23). Because most patients present with advanced metastatic disease, it is critical to understand the properties of invasive pancreatic tumors. Discovery and subsequent study of factors that contribute to tumor cell invasion provide an opportunity to develop therapies that could be used alone or in combination with other antineoplastic agents. Prior to our report (4), it was not previously known that QSOX1 was overexpressed in pancreatic tumors. The results presented in Fig. 1 suggest that QSOX1 is a commonly overexpressed protein in PDA, making it a potential target. To extend those initial findings, we began to investigate why pancreatic tumors overexpress QSOX1, and mechanistically, what advantage it affords tumors.

Tumor cells in which QSOX1 protein expression was suppressed by shQSOX1 grew more slowly than the shScrambled and untreated controls as measured by an MTT assay, whereas the results with our strongest shQSOX1, sh742, show a greater than 50% decrease in cell growth in both BxPC3 and Panc-1 cells (Fig. 2C). Our attempt to try and explain the decrease in proliferation as a result of abnormal cell-cycle regulation or an increase in apoptosis do not show a similar level of change that can solely explain our MTT results (Fig. 3; Supplementary data S2). Contrary to previous statements implicating QSOX1 as a cell-cycle regulator (24), our results suggest that although the loss of QSOX1 in Panc-1 cells shows a consistent decrease in G1, it is no where near that effect when we analyzed BxPC3 cells, suggesting that the role of QSOX1 could be cell type and tumor stage dependent, as a result of the different substrates available (Supplementary data S2). Our results likely conflict because we assessed the effect of QSOX1 on pancreatic tumor cell growth, not fibroblasts where QSOX1 was initially described (5, 24).

The same statement can be made in regards to the loss of QSOX1 directly affecting apoptosis. Although our strongest knockdown, sh742, does show at its greatest an 8% increase in Annexin V/PI double-positive cells, it is not enough to explain the dramatic decrease in cellular proliferation (Fig. 3). There are numerous proteins within the cell that assist in disulfide bond formation that may compensate for the loss of QSOX1 such as PDI, thioredoxin, glutathione, and members of the Erv family of sulfhydryl oxidases (25). There are no known preferred substrates of QSOX1, although speculation based on the function of QSOX1, as well as the known substrates that correspond to QSOX1 functional domains, leads us to believe that there are a broad spectrum of possible substrates, and, therefore, the role that QSOX1 plays in tumor cell progression would most likely be influenced by the substrates with the greatest affinity for QSOX1. Compensation by these other oxidases could help explain why the loss of QSOX1 does not lead to significant alterations in the cell cycle and apoptosis. It is also possible that suppression of QSOX1 activity does not induce apoptosis but results in other phenomena, such as anoikis or autophagy (26). We may investigate these possibilities in future studies.

Another hallmark of cancer is invasion. Because suppression of QSOX1 did not seem to play a major role in tumor cell growth, we hypothesized that the overexpression of QSOX1 in pancreatic tumor cells may contribute to their ability to degrade basement membranes, leading to an invasive and metastatic phenotype. We discovered that suppression of QSOX1 protein resulted in a dramatic reduction in the ability of both BxPC3 and Panc-1 pancreatic tumor cells to invade through Matrigel in vitro (Fig. 4). It is clear through these results that there are clear differences between BxPC3 and Panc-1 ability to degrade basement
membrane components and invade. This could be due to a myriad of factors such as the proteases secreted, the stage of the tumor, and genetic differences between the two cell lines (27). To determine whether this reasoning was correct, we carried out gelatin zymography as a way to analyze the MMP activity.

As a sulfhydryl oxidase, it is unlikely that QSOX1 would directly degrade basement membrane components.

Figure 5. Reduced expression of QSOX1 leads to a decrease in secreted pro-MMP-9 in BxPC3 and pro-MMP-2 Panc-1 cells. Gelatin zymography of (A) BxPC3 and (B) Panc-1 conditioned media showing a decrease in MMP-9 homodimers (MMP-9 complex; 240 and 130 kDa), pro-MMP-9 (92 kDa), pro-MMP-2 (72 kDa), and active MMP-2 (a-MMP-2, 66 kDa). Using ImageJ, we were able to quantify the percent decrease in pro-MMP-9 expression in BxPC3 (decrease in QSOX1, sh742–65%; sh528–47%; sh616–10%) and Panc-1 pro-MMP-2 (decrease in QSOX1, sh742–70%; sh528–56%; sh616–15%). C, Western blot analysis of MMP-2 and MMP-9 on conditioned serum-free media from shRNA-treated BxPC3 and Panc-1 cells. Full images can be seen in Supplementary Fig. S3. D, the effect of shRNA-mediated knockdown of QSOX1 on the expression of QSOX1-S, QSOX1-L, MMP-2, and MMP-9 in BxPC3 and (E) Panc-1 shRNA-treated cells was analyzed by quantitative real time PCR analysis. The graph represents relative gene expression calculated as ∆∆Cq using GAPDH as the endogenous reference gene. Significance, *, P < 0.05; **, P < 0.005.
Therefore, we hypothesized that MMPs serve as a substrate of QSOX1 while the MMPs are folding and undergoing activation as they are secreted from tumor cells. If true, suppression of QSOX1 would lead to a decrease in MMP functional activity, though not necessarily the amount of MMPs produced or secreted. Although the MMP profiles of BxPC3 and Panc-1 cells differ as seen in Fig. 5A and B, we found that suppression of QSOX1 leads to a decrease in pro-MMP-2 and MMP-9 activity. MMPs are zinc-dependent proteolytic enzymes that degrade ECM components (22). There are 23 known human MMPs as well as 4 known tissue inhibitors of MMPs (TIMPs) that aid in regulating the expression and activation of these proteolytic enzymes (22). The expression patterns of MMPs are variable depending on tumor type and even individual cell line.

In pancreatic cancer, the majority of MMPs are secreted in their inactive form and activated extracellularly (28). Activation of MMPs occurs either through the release of a covalent Cys^73-Zn^{2+} bond (“Cysteine Switch”) or through cleavage and activation by plasmin, serine proteases, and other MMPs or TIMPs (21, 28). MMP-2 and MMP-9 have been found to play an important role in pancreatic cancer progression with 93% of tumors expressing MMP-2 compared with normal tissue (28). Although reports implicating MMP-9 in the progression of pancreatic cancer are limited, Tian reported the proteomic identification of MMP-9 in pancreatic juice from patients with PDA (29). Pryczynicz and colleagues also found a relationship between MMP-9 expression and lymph node metastases (30). Numerous reports implicate MMP-2 as a prominent protease responsible for pancreatic tumor metastasis (22, 28).

One of the benefits of gelatin zymography is that it (a) provides a functional measure of the activities of MMPs able to degrade gelatin and (b) differentiates each precursor and active MMP by molecular weight (21, 31). A limitation of the zymography shown here is that it is limited to MMPs whose substrate is gelatin. It is possible that QSOX1 is involved in activation of other MMPs with different substrates. This will be investigated in future work.

Following up on our initial hypothesis about MMP activation by QSOX1, we conducted a Western blot analysis on the same serum-free conditioned media that was used to carry out gelatin zymography. Our result revealed that the overall levels of secreted MMP-2 and MMP-9 are nearly equal among the untreated, shScramble-, and shQSOX1-treated samples, leading us to further hypothesize that QSOX1 is involved in the proper folding of MMPs before they are secreted and that the loss of QSOX1 leads to proteolytically inactive MMPs as shown in Fig. 5A–C. To further confirm that what we are observing is a posttranslational event, we conducted qPCR on BxPC3 and Panc-1 shQSOX1-treated cells (Fig. 5D and E). Our observation was surprising in that we were able to show that there is an overall increase in the transcription of MMP-2 and MMP-9. This result led us to hypothesize that the cell is transcriptionally attempting to compensate for the proteolytically inactive MMPs through an as yet undetermined mechanism.

QSOX1 was previously reported by our group to be overexpressed in patients diagnosed with pancreatic cancer (4) and that a peptide from the QSOX1 parent protein is present in plasma from patients with PDA. In this study, we showed for the first time that expression of QSOX1 in pancreatic tumor cells directly contributes to an invasive and potentially metastatic phenotype through the activation of MMP-2 and MMP-9 through an as yet undetermined mechanism. It is not known whether QSOX1 is solely responsible for the proper folding of MMPs intracellularly, or whether it cooperates with protein disulfide isomerase while MMPs are folding in the ER and golgi. Because MMPs are secreted extracellularly, where they may undergo auto-activation or cleavage with proteases such as plasmin, it is possible that QSOX1-S activates them in the extracellular environment.

At this point, the posttranslational mechanism by which QSOX1 activates MMPs is not clear. Our results indicate that MMP-2 and MMP-9 RNA increased in shQSOX1-transduced cells. We expected no difference in MMP levels, but an increase might suggest that the cells are attempting to compensate for the lack of MMP activity through a feedback loop (32, 33). Although we hypothesize that QSOX1 may activate MMPs directly by involvement in the cysteine switch activation mechanism (21, 28), ROS produced by QSOX1 may be indirectly activating MMPs, as MMP activation has been reported to depend on an oxidative environment (32, 33).

Our results underscore the need to further understand the role that QSOX1 plays in tumor and normal cells, and how at the molecular level, it activates MMPs. This information will be useful during development of inhibitors of QSOX1 that may work upstream of individual MMPs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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QSOX1 Expression Promotes Tumor Cell Invasion

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Benjamin A. Katchman, Kwasi Antwi, Galen Hostetter, et al.


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