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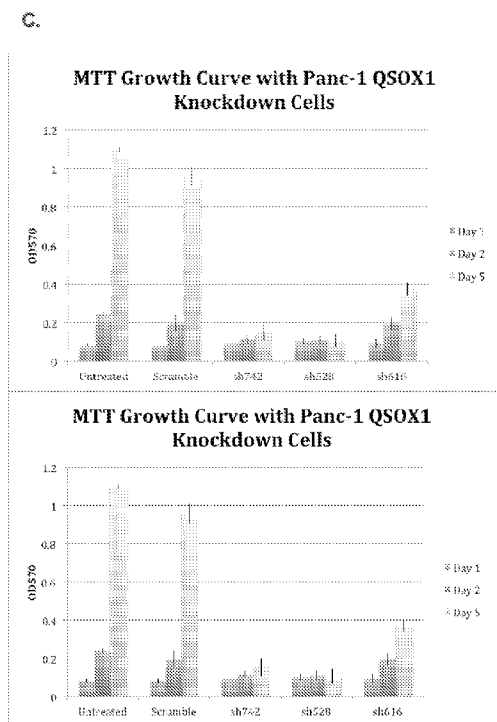
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(54) **Title:** QSOX1 AS AN ANTI-NEOPLASTIC DRUG TARGET

Figure 2

(57) **Abstract:** The present invention provides methods for tumor treatment by administering an inhibitor of quiescin sulfhydryl oxidase 1 (QSOX1), compositions comprising such inhibitors, and methods for identifying such inhibitors.

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MTT Growth Curve with Panc-1 QSOX1 Knockdown Cells

MTT Growth Curve with Panc-1 QSOX1 Knockdown Cells

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QSOX1 AS AN ANTI-NEOPLASTIC DRUG TARGET

Cross Reference

This application claims priority to US Provisional Patent Application Serial No. 61/384502 filed September 20, 2010, incorporated by reference herein in its entirety.

Background

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 pancreatic ductal adenocarcinoma have less than a 5% chance of surviving past five years (3).

Summary of the Invention

In a first aspect, the present invention provides methods for tumor treatment, comprising administering to a subject having a tumor an amount effective of an inhibitor of quiescin sulfhydryl oxidase 1 (QSOX1) expression and/or activity, or a pharmaceutically acceptable salt thereof, to treat the tumor. In one embodiment, the inhibitor of QSOX1 is selected from the group consisting of anti-QSOX1 antibodies, QSOX1-binding aptamers, QSOX1 antisense oligonucleotides, QSOX1 siRNA, and QSOX1 shRNA. In another embodiment, the tumor is a tumor that over-expresses QSOX1 compared to control. In a further embodiment, the subject is one from which tumor-derived QSOX1 peptides can be obtained. In a further embodiment, the tumor is a pancreatic tumor, and preferably a pancreatic adenocarcinoma. In a still further embodiment, the method is for limiting tumor metastasis.

In a second aspect, the present invention provides isolated nucleic acids, comprising or consisting of antisense, siRNA, miRNA, and/or shRNA molecules having a nucleic acid sequence that is perfectly complementary at least 10 contiguous nucleotides of QSOX1 as shown in SEQ ID NO: 1 and 2 or RNA equivalents thereof;

and/or fragments of the nucleic acid molecule.   In a preferred embodiment, the isolated nucleic acids comprising sequences from the group consisting of

5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID NO:11)

5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID NO:12),

5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID NO:13),

5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

In one embodiment of this second aspect of the invention, the isolated nucleic acids present in a short hairpin RNA (shRNA). In a further embodiment, the shRNA is of the general formula:

CCGG-X1-CTCGAGAAACTTTCTCACCACATTGGC
TTTTTG - 3' (SEQ ID NO: 23)

wherein X1 is a nucleic acid sequence selected from the group consisting of

5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID NO:11)

5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID NO:12),

5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID NO:13),

5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

2

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

In a third aspect, the present invention provides recombinant expression vectors comprising the isolated nucleic acid of any embodiment of the second aspect of the invention operatively linked to a promoter.

In a fourth aspect, the present invention provides recombinant host cells comprising the recombinant expression vector of the third aspect of the invention.

In a fifth aspect, the present invention provides pharmaceutical compositions, comprising

(a) the isolated nucleic acid of any embodiment of the second aspect of the invention, the recombinant expression vector of any embodiment of the third aspect of the invention, or the recombinant host cell of any embodiment of the fourth aspect of the invention; and

(b) a pharmaceutically acceptable carrier.

In a sixth aspect, the present invention provides methods for identifying candidate compounds for treating a tumor, comprising

(a) contacting tumor cells capable of expressing QSOX1 with one or more candidate compounds under conditions suitable for expression of QSOX1;

(b) determining a level of QSOX1 expression and/or activity in the tumor cells and comparing to control;

wherein a compound that decreases QSOX1 expression and/or activity in the tumor cells relative to control is a candidate compound for treating a tumor.

In one embodiment, the tumor cells are pancreatic tumor cells, preferably pancreatic adenocarcinoma cells.

Description of the Figures

Figure 1: QSOX1 is highly expressed in tumor cell lines but is not expressed in adjacent normal cells. Previously, our lab discovered a short peptide, NEQEQPLGQWHLS (SEQ ID NO: 3), in patient plasma through LCMS/ MS. 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 helix rich region (HRR).
QSOX1-L contains a predicted transmembrane (TM) domain. The peptide
NEQEQPLGQWHLS (SEQ ID NO: 3), maps back to QSOX1-L, and found to be

5   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 non-tumor cells. C.) Western blot analysis of

10   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 Adenocarinoma
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.

15   **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

20   – 46%; sh616 – 18%) cells and further evaluated cell growth, cell cycle, apoptosis,
and invasion/metastasis. C.) MTT assay on shRNA treated BxPC3 and Panc-1 cells
assayed on day 1, 2, and 5. Data represents averages ± standard deviation.
Significance *, P <0.05; **, P<0.01.

Figure 3: Reduced expression of QSOX1 in BxPC3 and Panc-1 cells leads to an

25   increase in annexin V/ propidium iodide positive cells. A.) Apoptosis Analysis
(Annexin V/Propidium Iodide) was performed on BxPC3 and Panc-1 cells in which
QSOX1 was reduced using shRNA. Plots show representative data from one of three
individual experiments for gated samples of Untreated, Scramble, sh742, sh528 and
sh616. The percentages represent the number of cells that are annexin V positive

30   (Lower Right), annexin V/propidium iodide double positive (Upper Left), or
propidium iodide positive (Upper Right). Data was calculated using Cell Quest Pro
software.

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 shRNA's specific for QSOX1 and seeded in the top chamber of Matrigel^{TM} invasion wells and allowed to incubate for 18 hours. Representative 10x, 20x, and 40x 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^{TM} and invade to the underside of the membrane, respectively. While 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^{TM} and invade through the membrane. Graphs represent average ± standard deviation (BxPC3 n = 6; PANC-1 n = 3), significance *, $P < 0.05$, **, $P < 0.005$.

Figure 5: Reduced expression of QSOX1 leads to a decrease in secreted proMMP-9 in BxPC3 and proMMP-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-MMP9 (92 kDa), pro-MMP2 (72 kDa) and active MMP-2 (a-MMP2, 66 kDa). Using Image J we were able to quantify the percent decrease in proMMP-9 expression in BxPC3 (Decrease in QSOX1, sh742 –65%; sh528 – 47%; sh616 – 10%) and Panc-1 proMMP-2 (Decrease in QSOX1, sh742 – 70%; sh528– 56%; sh616 – 15%). C.) Western blot analysis of MMP-2 and -9 on conditioned serum free media from shRNA treated BxPC3 and Panc-1 cells. 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 Panc-1 shRNA treated cells was analyzed by quantitative real time PCR analysis. The graph represents relative gene expression calculated as ΔΔCq using GAPDH as the endogenous reference gene.

Figure 6. Cellular proliferation was measured by MTT assay 4 days post-transfection shows that knockdown of QSOX1 short and long form expression by transient lipid-mediated transfection of shRNA results in a decrease in tumor cell viability as measured by a cellular mitochondrial respiration assay (MTT assay). This supports the idea that shRNA or other RNAi species could be used as a drug to suppress QSOX1 protein expression, and inhibit the growth, invasion, and metastases of tumors over-expressing QSOX1. **No shRNA** – Untreated BxPC3 Cells; **Scrambled shRNA** – Negative control. BxPC3 cells transfected with scrambled shRNA control vector; **shRNA1** – BxPC3 cells transfected with shRNA1 for 4 days; **shRNA2** – BxPC3 cells transfected with shRNA2 for 4 days.

Figure 7. Invasion Assay shows that by knocking down QSOX1 with the above shRNA we are able to see a decrease in tumor cell invasion. Cells were transfected, allowed to recover for 4 days, resuspended in serum free media and added to 8um (pore size) microwell inserts coated with Matrigel™. The inserts were placed in cell

5   culture media containing 10% fetal bovine serum such that the bottom half of the outside of the insert was exposed to the media. After 24 hours incubation at 37°C, 5% CO_2, the inserts were removed and washed in buffer. Cells that were able to degrade the Matrigel™ coating and invade through the 8um pores in the insert were counted on the underside of the well.

10   Using the Matrigel™ assay we are able to show that when we knock down QSOX1 in BxPC3 (pancreatic cancer cells) the cells are no longer able to degrade the basement membrane components resulting in a decrease in cellular invasion. **Untreated** – Untreated BxPC3 Cells; **Scrambled** – BxPC3 cells transfected with scrambled shRNA control vector. Invasion measured at 4 days post-transfection; **shRNA1** –

15   BxPC3 cells transfected with shRNA1 and invasion measured 4 days post-transfection; **shRNA2** – BxPC3 cells transfected with shRNA2 and invasion measured 4 days post-transfection; **shRNA1/2** – BxPC3 cells transfected with a combined shRNA1 and shRNA2. Cells were measured for invasion properties 4 days post-transfection.

20

Detailed Description of the Invention

All references cited are herein incorporated by reference in their entirety. Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory*

25   *Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego,

30   CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. "And" as used herein is interchangeably used with "or" unless expressly stated otherwise.

All embodiments of any aspect of the invention can be used in combination, unless the context clearly dictates otherwise.

In a first aspect, the present invention provides methods for tumor treatment, comprising contacting a subject having a tumor with an amount effective of an inhibitor of QSOX1 expression and/or activity, or pharmaceutically acceptable salt thereof, to treat the tumor.

As demonstrated in the examples that follow, the inventors of the present invention have discovered that inhibitors of QSOX1 expression and/or activity of QSOX1 can be used to treat tumors.

As used herein, "QSOX1" is Quiescin Sulfhydryl Oxidase 1, also called QSCN6. The protein accession number for the long variant of QSOX1 on the NCBI database is NP_002817 (SEQ ID NO:24), and the accession number for the short form is NP_001004128 (SEQ ID NO:25). As used herein, "QSOX1" refers to both the long and short variants of QSOX1.

The subject can be any mammal, preferably a human.

As used herein, any suitable "inhibitor of QSOX1 expression and/or activity" can be used that is capable of reducing expression of QSOX1 mRNA expression or protein synthesis, or that can inhibit activity of QSOX1 protein via any mechanism, including but not limited to binding to QSOX1 resulting in inhibition of QSOX1 activity. Such inhibitors can include, but are not limited to small molecules, antibodies, and aptamers that inhibit activity of QSOX1 protein, and antisense, siRNA, shRNA, etc. that inhibit expression of QSOX1 mRNA and/or protein. Inhibitors of QSOX1 expression may be identified through any suitable means, including but not limited to the methods described below. Any suitable method for determining QSOX1 activity levels may be used, including but not limited to those described in detail below.

As used herein, "inhibit" means at least a 10% reduction in QSOX1 expression and/or activity; preferably at least a 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater reduction in expression and/or activity.

The methods of the invention can be used to treat any suitable tumor type. In one preferred embodiment, the methods are used to treat any tumor type that over-

expresses QSOX1. Expression of QSOX1 can be assessed by any suitable method, including but not limited to immunohistochemistry of suitable tissue sample, polymerase chain reaction, or detection of QSOX1 peptides in suitable tissue, as disclosed, for example, in WO 2010/071788; WO 2010/01787; and WO

5   2010/077921, incorporated by reference herein in their entirety. In various non-limiting embodiments, techniques that can be used in the analysis include mass spectrometry (MS), two dimensional gel electrophoresis, Western blotting, immunofluorescence, ELISAs, antigen capture assays (including dipstick antigen capture assays) and mass spec immunoassay (MSIA). In one preferred embodiment,

10   ligands for the one or more peptides are used to "capture" antigens out of the tissue sample. Such ligands may include, but are not limited to, antibodies, antibody fragments and aptamers. In one embodiment, the ligand(s) are immobilized on a surface and the sample is passed over the surface under conditions suitable for binding of any peptides in the sample to the ligand(s) immobilized on the surface.

15   Such antigen capture assays permit determining a concentration of the peptides in the tissue sample, as the concentration likely correlates with extent of disease.

The tissue sample may be any suitable sample from which tumor-derived peptides may be obtained. In various preferred embodiments, the tissue sample is selected from the group consisting of plasma, serum, urine, saliva, and relevant tumor

20   tissue. In various preferred embodiments for detecting QSOX1 peptides in tissue (preferably plasma or serum), the peptide to be detected is selected from the group consisting of NEQEQPLGQWHLS (SEQ ID NO: 3), NEQEQPLGQWH (SEQ ID NO: 4), EQPLGQWHLS (SEQ ID NO: 5), AAPGQEPPEHMAELQR (SEQ ID NO: 6), AAPGQEPPEHMAELQ (SEQ ID NO: 7),

25   AAPGQEPPEHMAELQRNEQEQPLGQWHLS (SEQ ID NO: 8), NEQEQPL (SEQ ID NO:9), and GQWHLS (SEQ ID NO: 10).

As used herein, the phrase "an amount effective" refers to the amount of inhibitor that provides a suitable treatment effect.

Any suitable control can be used to compare with QSOX1 expression and/or

30   activity in the subject's tissue. In one embodiment, the control comprises an amount of one or more peptides of interest from a tissue sample from a normal subject or population (ie: known not to be suffering from a tumor), or from a subject or population of subjects suffering from a tumor, using the same detection assay. The control tissue sample will be of the same tissue sample type as that assessed from the

test subject. In one preferred embodiment, a standard concentration curve of one or more peptides of interest in the control tissue sample is determined, and the amount of the one or more peptides of interest in the test subject's tissue sample is compared based on the standard curve. In another preferred embodiment for use in monitoring

5   progress of tumor therapy, samples are obtained from patients over time, during or after their therapy, to monitor levels of one or more peptides in plasma as an indication about tumor burden in patients. The control may comprise a time course of concentration of the one or more peptides of interest in a given tissue type of the test subject, to monitor the effect of treatment on the concentration; this embodiment is

10   preferred, for example, when assessing efficacy of tumor treatments. Those of skill in the art will recognize that similar controls can be used for immunohistochemical-based analysis. Based on the teachings herein and the knowledge in the art, those of skill in the art can design a variety of other appropriate controls in assessing QSOX1 expression and/or activity in identifying subjects most likely to respond to the

15   treatment methods of the invention, as well as to assess efficacy of the treatment over time.

Thus, in one preferred embodiment, the method comprises identifying subjects with tumors that over-express QSOX1, and treating such patients according to the methods of the invention. In one preferred embodiment, the method comprises

20   measuring QSOX1 expression in blood plasma to identify tumors that over-express QSOX1. Methods for preparing blood plasma are well known in the art. In one embodiment, plasma is prepared by centrifuging a blood sample under conditions suitable for pelleting of the cellular component of the blood.

Non-limiting tumor types that can be treated using the methods of the

25   invention include pancreatic, lung, colon, breast, and prostate tumors. In a preferred embodiment, the tumor is a pancreatic tumor, such as a pancreatic adenocarcinoma or a neuroendocrine tumor. In a further preferred embodiment, the tumor comprises a pancreatic adenocarcinoma.

As used herein, "treating tumors" means accomplishing one or more of the

30   following: (a) reducing tumor mass; (b) slowing the increase in tumor mass; (c) reducing tumor metastases; (d) slowing the incidence of tumor metastases; (e) limiting or preventing development of symptoms characteristic of cancer; (f) inhibiting worsening of symptoms characteristic of cancer; (g) limiting or preventing recurrence of symptoms characteristic of cancer in subjects that were previously

symptomatic; (i) increasing subject survival time; and (j) limiting or reducing morbidity of therapy by enhancing current therapies, permitting decreased dose of current standard of care therapies.

5   For example, symptoms of pancreatic cancer include, but are not limited to, pain in the upper abdomen, significant weight loss, loss of appetite and/or nausea and vomiting, jaundice, and Trousseau sign. In a preferred embodiment, the methods limit tumor metastasis, such as limiting pancreatic tumor metastasis. As shown in the examples that follow, the inventors have shown that knockdown of QSOX1 expression in tumor cells leads to a dramatic decrease in tumor cell

10   invasive/migratory phenotype, thus making the methods of the invention particularly useful for limiting tumor metastasis. While not being limited by any particular mechanism of action, the inventors believe that this inhibition of metastasis may result from a decrease in the proteolytic activity of matrix metalloproteases 2 and 9 (MMP-2 and MMP-9), as discussed in more detail in the examples that follow.

15   In a preferred embodiment of all of the above embodiments, the inhibitor is selected from the group consisting of antibodies, antisense RNA, siRNA, miRNA, and shRNA. In a further preferred embodiment, the inhibitor comprises or consists of antisense, siRNA, miRNA, and/or shRNA molecules having a nucleic acid sequence perfectly complementary to least 10 contiguous nucleotides of QSOX1 as shown in

20   SEQ ID NO:1 and 2 or RNA equivalents thereof; and/or fragments of the nucleic acid molecule. In various preferred embodiments, the nucleic acid molecule is perfectly complementary to at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more contiguous nucleotides of QSOX1, or an RNA equivalent thereof.

In another preferred embodiment, the inhibitor comprises or consists of a

25   nucleic acid selected from the group consisting of
5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID NO: 11)
5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID NO:12),

30   5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID NO:13),
5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14),
5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15),
and

5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16).

wherein residues noted as "(T/U)" can be either "T" or "U'. In further

preferred embodiments, the inhibitor comprises or consists of

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

In a further preferred embodiment, the nucleic acids of SEQ ID NO:11-22 are

part of a short hairpin RNA (shRNA). In this embodiment, such an shRNA comprises

flanking regions, loops, antisense and spacer sequences flanking a recited SEQ ID

NO: sequence responsible for the specificity of shRNA. There are no specific

sequence requirements for these various other shRNA regions so long as a loop

structure can be formed. Exemplary constructs are shown in the examples below.

ShRNA are thought to assume a stem-loop structure with a 2 nucleotide 3' overhang

that is recognized by Dicer and processed in to siRNA. SiRNA are then recognized

by RNA-Induced Silencing Complex (RISC) which removes the sense strand from the

stem structure leaving the guide strand allowing it to associate with target mRNA and

cleave it: QSOX1, in this case. Full length protein cannot be translated after cleavage.

In one exemplary embodiment, the shRNA comprise or consist of a nucleic

acid of the general formula:

CCGG-X1-CTCGAGAAACTTTCTCACCACATTGGCTTTTG - 3' (SEQ

ID NO: 23)

wherein X1 is a nucleic acid sequence selected from the group consisting of

5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID

NO:11)

5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID

NO:12),

5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID

NO:13),

5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

5   5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

These and other nucleic acid inhibitors may be modified for a desired purpose, including but not limited to nucleic acid backbone analogues including, but not limited to, phosphodiester, phosphorothioate, phosphorodithioate,

10   methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, peptide nucleic acids (PNAs), methylphosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) Biochemistry 36:8692-8698), and benzylphosphonate linkages, as discussed in US 6,664,057; see also Oligonucleotides

15   and Analogues, a Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) J. Med. Chem. 36:1923-1937; Antisense Research and Applications (1993, CRC Press). Nucleic acid inhibitors may also comprise analogous forms of ribose or deoxyribose

20   as are well known in the art, including but not limited to 2' substituted sugars such as 2'-O-methyl-, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α.-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. The oligonucleotides may also contain TNA (threose nucleic acid; also

25   referred to as alpha-threofuranosyl oligonucleotides) (See, for example, Schong et al., Science 2000 Nov. 17, 290 (5495):1347-1351.)

The inhibitors for use in the present invention can be administered via any suitable technique or formulation, including but not limited to lipid, virus, polymer, or any other physical, chemical or biological agent, but are generally administered as

30   part of a pharmaceutical composition together with a pharmaceutically acceptable carrier, diluent, or excipient. Such compositions are substantially free of non-pharmaceutically acceptable components, *i.e.*, contain amounts of non-pharmaceutically acceptable components lower than permitted by US regulatory requirements at the time of filing this application. In some embodiments of this

aspect, if the compound is dissolved or suspended in water, the composition further optionally comprises an additional pharmaceutically acceptable carrier, diluent, or excipient. In other embodiments, the pharmaceutical compositions described herein are solid pharmaceutical compositions (*e.g.*, tablet, capsules, *etc.*). The isolated nucleic acids or shRNAs can be present in a vector, such as a viral vector (ex: retrovirus, lentivirus, adenovirus, adeno-associated virus, etc.), for delivery via any suitable technique.

These compositions can be prepared in a manner well known in the pharmaceutical art, and can be administered by a variety of routes, depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be via physical injection with a needle to, for example, a tumor in the subject; topical (including ophthalmic and to mucous membranes including intranasal, vaginal and rectal delivery), pulmonary (*e.g.*, by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), ocular, oral or parenteral. Methods for ocular delivery can include topical administration (eye drops), subconjunctival, periocular or intravitreal injection or introduction by balloon catheter or ophthalmic inserts surgically placed in the conjunctival sac. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, *e.g.*, intrathecal or intraventricular, administration. Parenteral administration can be in the form of a single bolus dose, or may be, for example, by a continuous perfusion pump. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Also, pharmaceutical compositions can contain, as the active ingredient, one or more inhibitors described herein above in combination with one or more pharmaceutically acceptable carriers, and may further comprise one or more additional active agents as appropriate for a given therapeutic treatment. In making the compositions described herein, the active ingredient is typically mixed with an excipient, diluted by an excipient or enclosed within such a carrier in the form of, for example, a capsule, sachet, paper, or other container. When the excipient serves as a diluent, it can be a solid, semi-solid, or liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of

tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

5   Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, and methyl cellulose. The formulations can additionally include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents;

10   emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxy-benzoates; sweetening agents; and flavoring agents. The compositions described herein can be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the patient by employing procedures known in the art.

15   The compositions can be formulated in a unit dosage form, each dosage containing from about 1 to about 1000 mg, more usually about 10 to about 500 mg, of the active ingredient. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the

20   desired therapeutic effect, in association with a suitable pharmaceutical excipient. It will be understood, however, that the amount of the compound actually administered will usually be determined by a physician, according to the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual inhibitor administered, the age, weight, and response of the individual patient, the

25   severity of the patient's symptoms, and the like.

The inhibitors described herein can also be formulated in combination with one or more additional active ingredients as desired.

The present invention also provides methods for limiting tumor metastasis, comprising contacting a subject having a tumor with an amount effective of an

30   inhibitor of QSOX1 expression and/or activity, or pharmaceutically acceptable salt thereof, to limit metastasis of the tumor in the subject. As shown in the examples that follow and as discussed above, QSOX1 inhibitors slow tumor growth and inhibit the metastatic process.

All embodiments of the first aspect of the invention are equally applicable to this second aspect, unless the context clearly dictates otherwise. As used herein, "limiting metastasis" means any limitation over what would be seen in the absence of administration of the QSOX1 inhibitor. In a preferred embodiment, limiting

5   metastasis comprises a statistically significant limitation compared to control subjects not treated with the QSOX1 inhibitor. In another preferred embodiment, the tumor comprises a pancreatic tumor; even more preferably a pancreatic adenocarcinoma.

In a second aspect, the present invention provides isolated nucleic acids, comprising or consisting of antisense, siRNA, miRNA, and/or shRNA molecules

10   having a nucleic acid sequence perfectly complementary to least 10 contiguous nucleotides of QSOX1 as shown in SEQ ID NO:1 and 2 or RNA equivalents thereof; and/or fragments of the nucleic acid molecule. In various preferred embodiments, the nucleic acid molecule is perfectly complementary to at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more contiguous nucleotides of QSOX1, or an RNA

15   equivalent thereof.

In a further preferred embodiment, the isolated nucleic acids comprise or consist of a nucleotide sequence selected from the group consisting of

5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID NO:11)

20   5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID NO:12),

5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID NO:13),

5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

25   5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

30   5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

The isolated nucleic acids can be used, for example, in the methods of the invention. The isolated nucleic acids of this second aspect of the present invention can be modified as described above in the first aspect of the invention, including nucleic acid backbone analogues and analogous forms of ribose or deoxyribose.

5   As used herein, "isolated" means that the nucleic acids are removed from their normal surrounding nucleic acid sequences in the genome or in cDNA sequences, and are substantially free of contaminating material used to isolate them (ie: agarose, polyacrylamide, column chromatography resins, and the like). The isolated nucleic acids may be stored in any suitable state, including but not limited to in solution or as

10   a lyophilized powder.

The isolated nucleic acids may be chemically synthesized using means known in the art, or may be generated by recombinant expression vectors.

As used herein, the isolated nucleic acids "comprising" the recited nucleotide sequences means that the recited nucleotide sequences can be present as part of a

15   larger synthetic construct, such as an antisense nucleic acid, siRNA, an shRNA, miRNA, or as part of a construct in association (covalently bound or non-covalently bound) with a lipid, virus, polymer, or any other physical, chemical or biological agent. As used herein, the isolated nucleic acids "comprising" the recited nucleotide sequences does not include the isolated nucleic acid as part of a naturally occurring or

20   isolated full length QSOX1 transcript or cDNA thereof.

In one embodiment, the isolated nucleic acid is present in a short hairpin RNA (shRNA). As discussed above, such an shRNA comprises flanking regions, loops, antisense and spacer sequences flanking a recited SEQ ID NO: sequence responsible for the specificity of shRNA. There are no specific sequence requirements for these

25   various other shRNA regions so long as a loop structure can be formed. Exemplary constructs are shown in the examples below. In one embodiment, the shRNA is of the general formula: CCGG-X1-CTCGAGAAACTTTCTCACCACATTGGC TTTTTG - 3' (SEQ ID NO: 23)

wherein X1 is a nucleic acid sequence selected from the group consisting of

30   5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID NO:11)

5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID NO:12),

5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID
NO:13),

5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

5   5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

10   5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21; and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

In a third aspect, the present invention provides recombinant expression
vectors comprising the isolated nucleic acid of any embodiment of the third aspect of
15   the invention operatively linked to a promoter. "Recombinant expression vector"
includes vectors that operatively link a nucleic acid coding region or gene to any
promoter capable of effecting expression of the gene product. The vectors can be
used, for example, for transfection of host cells for large scale production of the
isolated nucleic acids, or may be used as vector delivery systems in the methods of the
20   invention. The promoter sequence used to drive expression of the disclosed nucleic
acid sequences in a mammalian system may be constitutive (driven by any of a
variety of promoters, including but not limited to, utilizes the U6 or H1 promoter
promoter (to ensure that the shRNA is always expressed), CMV, SV40, RSV, actin,
EF) or inducible (driven by any of a number of inducible promoters including, but not
25   limited to, tetracycline, ecdysone, steroid-responsive). The construction of expression
vectors for use in transfecting eukaryotic and prokaryotic cells is also well known in
the art, and thus can be accomplished via standard techniques. (See, for example,
Sambrook, Fritsch, and Maniatis, in: Molecular Cloning, A Laboratory Manual, Cold
Spring Harbor Laboratory Press, 1989; *Gene Transfer and Expression Protocols*, pp.
30   109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion
1998 Catalog (Ambion, Austin, TX). The expression vector must be replicable in the
host organisms either as an episome or by integration into host chromosomal DNA.
In a preferred embodiment, the expression vector comprises a viral vector or a

plasmid. Any suitable viral vector may be used, including but not limited to retroviruses, lentiviruses, adenoviruses, adeno-associated viruses, etc.

In a fourth aspect, the present invention provides host cells comprising the recombinant expression vectors disclosed herein, wherein the host cells can be either

5   prokaryotic or eukaryotic. The host cells can be used, for example, in large scale production of the recombinant vectors of the invention. The cells can be transiently or stably transfected if a plasmid vector is used, or may be transiently or stably transduced when a viral vector is used. Such transfection and transduction of expression vectors into prokaryotic and eukaryotic cells can be accomplished via any

10   technique known in the art, including but not limited to standard bacterial transformations, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. (See, for example, *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press; *Culture of Animal*

15   *Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY).

In a fifth aspect, the present invention provides pharmaceutical compositions, comprising

(a)   the isolated nucleic acid of any embodiment of the second aspect of the

20   invention, the recombinant expression vector of any embodiment of the third aspect of the invention, or the recombinant host cell of any embodiment of the fourth aspect of the invention; and

(b)   a pharmaceutically acceptable carrier.

The pharmaceutical compositions can be used, for example, in the methods of

25   the invention. As used herein, the phrase "pharmaceutically acceptable salt" refers to both pharmaceutically acceptable acid and base addition salts and solvates. Such pharmaceutically acceptable salts include salts of acids such as hydrochloric, phosphoric, hydrobromic, sulfuric, sulfinic, formic, fumaric, toluenesulfonic, methanesulfonic, nitric, benzoic, citric, tartaric, maleic, hydroiodic, alkanoic such as

30   acetic, $HOOC\text{-}(CH_2)_n\text{-}COOH$ where n is 0-4, and the like. Non-toxic pharmaceutical base addition salts include salts of bases such as sodium, potassium, calcium, ammonium, and the like. Those skilled in the art will recognize a wide variety of non-toxic pharmaceutically acceptable addition salts. All embodiments of

pharmaceutical compositions discussed herein for the methods of the invention can be used in this aspect of the invention.

In a sixth aspect, the present invention provides methods for identifying candidate compounds for treating a tumor, comprising

(a)   contacting tumor cells capable of expressing QSOX1 with one or more candidate compounds under conditions suitable for expression of QSOX1;

(b)   determining a level of QSOX1 expression and/or activity in the tumor cells and comparing to control;

wherein a compound that decreases QSOX1 expression and/or activity in the tumor cells relative to control is a candidate compound for treating a tumor.

Any tumor cell can be used that is capable of expressing QSOX1, either inherently or as a result of transfecting the cell with a QSOX1 recombinant expression vector. In a preferred embodiment, the tumor cell is selected from the group consisting of pancreatic, lung, colon, breast, and prostate tumor cells. In a preferred embodiment, the tumor cells are pancreatic tumor cells, preferably pancreatic adenocarcinoma cells.

Any suitable candidate compound can be used, including but not limited to small molecules, antibodies, aptamers, antisense, siRNA, and shRNA.

As used herein, "inhibit" means at least a 10% reduction in expression and/or activity; preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater reduction in expression and/or activity.

Any suitable control can be used, including but not limited to tumor cells not treated with test compound, and average expression product levels of QSOX1 in a control cell population. In one embodiment, the control comprises a cell from a normal subject or population (ie: known not to be suffering from a tumor), or from a subject or population of subjects suffering from a tumor, using the same detection assay. In another preferred embodiment, the control comprises a tumor cell contacted with the isolated nucleic acid, shRNA, or expression vector of the invention, to facilitate identifying compounds with increased QSOX1 inhibitory activity than the nucleic acids disclosed herein. The control cell will be of the same type as that assessed from the test subject. In one exemplary embodiment where immunohistochemistry is used to assess QSOX1 expression, a suitable control is normal tissue in a pathological sample.

Any suitable method for determining QSOX1 expression levels may be used, including but not limited to reverse transcription-polymerase chain reaction (RT-PCR), Western blot, in situ hybridization, and immunohistochemical analysis (such as fluorescence in situ hybridization)

5   Similarly, any suitable method for determining QSOX1 activity levels may be used, including but not limited to an oxygen electrode assay, using the enzymatic activity of QSOX1 to detect structures/compounds that inhibit the ability of QSOX1 to oxidize a known substrate.

Example 1

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 pancreatic ductal adenocarcinoma have less than a 5% chance of surviving past five years (3). Through proteomic analysis of pancreatic cancer patient plasma, we discovered a peptide from QSOX1 that maps back to the C-terminus of the long isoform of QSOX1 (QSOX1-L) (4). Subsequently, we found that QSOX1 is over-expressed in tumor tissue from pancreatic cancer patients, but not adjacent normal tissue (Fig. 1B & C). These findings led us to hypothesize that over-expression 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 sulfhydryl 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 sulfhydryl 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) (10). Both, QSOX1-S and -L have identical functional domain organization from the amino terminus as follows: two thioredoxin-like domains (Trx1 &2), a helix rich region (HRR) and an Erv/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) (12). QSOX1 was originally discovered in quiescent human lung fibroblasts and was hypothesized to aid in the transition from G_0 to S phase of the cell cycle (13, 14). Thorpe et al. revealed the ability of QSOX1 to efficiently generate disulfide bonds into proteins during folding at rate of 1000 per minute with a K_M of 150uM per thiol (7). QSOX1 appears 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.

21

In the present 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 –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,

5   apoptosis, invasion and matrix metalloproteinase activity. QSOX1 knock-downs 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 report demonstrating a

10   role for QSOX1 in invasion and metastasis.

Material and Methods

Cell culture

Pancreatic adenocarcinoma BxPC3, PANC-1, CFPac-1, and Capan1 cancer

15   cell lines were cultured in DMEM with 10% fetal bovine serum (FBS) (Gibco). Immortal human non-tumorigenic pancreatic duct epithelial cells (HPDE6) were cultured in Clontech KGM-2 karotinocyte media (Gibco) (19). All cell lines were grown at 37^0C with 5% CO_2. Cell lines are tested monthly for mycoplasma contamination using, Venor GeM™ Mycoplasma Detection Kit, PCR based from

20   Sigma.

Immunohistochemistry (IHC)

Immunohostochemistry on patients who underwent surgical resection was performed in the exact same manner as previously described in Kwasi et al (4).

25   **Generation of Short hairpin (sh) RNA and Lentiviruses Production**

Three different shRNA for QSOX1 were obtained through DNASU (http://dnasu.asu.edu)(20) already in the lentiviral pLKO.1-puromycin selection vector. QSOX1 sh742, 5' – CCGG*GCCAATGTGGTGAGAAAGTTT*CTCGAGAAACTTT

30   CTCACCACATTGGCTTTTTG - 3' (SEQ ID NO: 26)(sense), QSOX1 sh528, 5'- *CCGGACAATGAAGAAGCCTTT* - 3' (SEQ ID NO: 27) (sense), QSOX1 sh616, 5'- *TCTAGCCACAACAGGGTCAAT* -3' (SEQ ID NO: 28) (sense) and shScramble with target sequence

5' –*TCCGTGGTGGACAGCCACATG* – 3' (SEQ ID NO: 29) was obtained from Josh
LaBaer's laboratory at Arizona State University. The target sequence is underlined
and each vector contains the same supporting sequence surrounding the target
sequence.

5   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% CO_2 for 24
hrs. The following day the 293T cells were transfected with 2500ng shRNA maxi-
prepped plasmid DNA (Sigma GeneElute™ HP Plasmid Maxiprep Kit), 500ng
10   VSVg, 2500ng d8.91(gag-pol) in LT1 transfection reagent from Mirus Bio (Madison,
WI) and centrifuged at 1000g for 30 minutes and incubated as 37°C, 5% CO_2 for 24
hrs at in media lacking antibiotics. The next morning media containing lentivirus was
collected and replaced with complete media. Supernatants (2.5ml) from transfected
293T cells producing each lentivirus were collected every 24 hours for a total of 72
15   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 performed by first seeding the cells at 8×10^5 cells/well in a 6
20   well plate and incubating overnight. The next day the cells were transduced by adding
8ug/mL polybrene (Millipore) and 200ul sh742, sh528, sh616 and shScramble
lentivirus media from 293T cells to each well. The cells were spun at 1000 rpm for 30
minutes and then incubated for 24 hours. The following day fresh DMEM with 10%
FBS was added, containing 1ug/mL puromycin (Sigma), to select for the transduced
25   cells QSOX1 knockdown was measured by western blot.

SDS-PAGE-Western blotting

Western blotting was performed using cell lysates from HPDE6, BxPC3,
Panc-1, Capan1 and CFPac1 cells as well as patient 1010 and 1016 tumor and
30   adjacent normal enzymatic supernatant. Cell lysates were generated by harvesting 2.5
$\times 10^6$ cells by centrifugation followed by lysis using RIPA buffer (50mM Tris-HCl,
pH 7.4, 150mM NaCl, 1mM EDTA, and 1% Triton X-100) with 1x 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 2ug/mL (20ug total protein per lane). Samples were run on 10% SDS-polyacrylamide gels then transferred onto Immun-Blot™ PVDF Membranes (Bio-Rad). Rabbit polyclonal anti-QSOX1 (ProteinTech), rabbit polyclonal anti-Bactin and anti-alpha-tubulin (Cell Signaling), and rabbit polyclonal

5   anti-MMP-2 and -9 (Sigma) antibody was diluted 1:1000, 1:1000, and 1:500 respectfully, in 0.1% BSA in 1x TBS+ 0.01% Tween-20 and incubated for overnight. Goat anti-rabbit IgG-alkaline phospatase or HRP secondary antibody was used at a 1:5000 dilution and incubated with the blot for 1 h followed by washing. BCIP/NBT substrate (Pierce Chemical, Rockford, IL) was added and the blot was developed at

10   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 Image J and is presented as percent change from the scrambled shRNA control. Full gel images are

15   available in the supplemental material. All gel images were annotated and processed using Photoshop software.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

Cells were seeded at 3×10^3 cell/well in a 96-well plate in triplicate and

20   incubated at 37°C, 5% CO_2 over the course of 5 days. The MTT assay was performed on days 1, 2 and 5 according to the manufacturers instructions (Invitrogen-Molecular Probes, Vybrant MTT Cell Proliferation Assay Kit). Results are presented as mean +/- S.D. Student's two tailed T-test was performed to determine significance.

25   **Annexin V / Propidium Iodide Apoptosis Analysis**

Apoptosis analysis was performed according to the manufacturer's instructions (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen). Briefly, cells were seeded at equal densities in a $25cm^2$ flask until they reached 60-80% confluency. The cells were then washed with cold PBS, counted, and normalized to

30   1×10^6 cell/ml in 1x Annexin V Binding Buffer. Next, 1×10^5 cells were then transferred to a separate tube and 5μl of FITC Annexin V and 5μl of Propidium Iodide were added to each sample. The samples were gently vortexed and incubated for 15 min at RT in the dark. Lastly, 400μl of 1x Binding Buffer was added to each sample

and the samples were analyzed by flow cytometer (Becton Dickinson FACScalibur™ Flowcytometer) with 1 hr. Each sample was performed in triplicate.

Gelatin Zymography

The identification of matrix metalloproteinases (MMP) was performed using gelatin zymography. Zymography experiments were performed as follows. Untreated BxPC3 and Panc-1 cells as well as transduced cells were seeded at 5 x 10^5 cells/well (12 well plates) in DMEM with 10% FBS. The next day, cells were then washed with 1xPBS 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 performed with a 10% polyacrylamide gel containing gelatin solution in place of water (0.8 mg/mL Gelatin, 0.15 M Tris pH 8.8, 30% acrylamide-bis, 50% glycerol, 10% SDS, 10% APS, and TEMED) (21). A volume of equal concentrations of serum free conditioned media were loaded under non-denaturing 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 performed at a constant voltage of 150 V for 60 min. Gels were washed in renaturing buffer (25% Triton X-100 in water) for 30 min at RT with gentle shaking. The gels were then equilibrated in developing buffer (50 mM Tris-base, 6.3 g/L Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% Triton X-100) for 30 min at RT with gentle shaking. Fresh developing buffer was then added to the gels and they were incubated overnight at 37^0C. The gels were then stained with SimplyBlue™ Safe Stain (Invitrogen) for 20 minutes at RT, then destained overnight in ddH_2O at RT. The presence of MMP was detected by the lack of staining indicating digestion of gelatin. The negative control was performed by adding, 50 mM Ethylene Diamine Tetra Acetic Acid (EDTA), to both the renaturing buffer and the developing buffer to block the MMP activation. Quantification of band intensity was measured using Image J and is presented as percent change from the scrambled shRNA control.

RNA Isolation and cDNA Synthesis

Total RNA isolation was performed according to the manufactures instructions for animal cells using spin technology (RNeasy™ Mini Kit, Qiagen). After RNA was isolated from each sample was reverse transcribed with qScript™ cDNA Sythesis Kit, Quanta Biosciences according to the manufactures instructions.

Quantitative Real Time PCR (qPCR)

The relative level of GAPDH, QSOX1-L, QSOX1-S, MMP-2, and MMP-9 were analyzed in each sample by qPCR. Each cDNA sample was normalized to
5   100ng/µl in molecular grade water along with 100nM final concentration of each primer and 1x final concentration of PerfeCta™ SYBR Green Fast Mix, ROX to a final volume of 20µl. qPCR was performed using, PerfeCTa™ SYBR Green FastMix, ROX from Quanta Biosciences on a ABI7900HT thermocycler, Applied Biosystems Inc. Reaction Protocol: Initial Denaturation – 95°C for 3 min; PCR Cycling (40
10   cycles) 1.) 95°C, 30 sec. 2.) 55°C, 30 sec. 3.) 72°C, 1 min; Melt Curve (Dissociation Stage). The primer sequences for the genes analyzed are: GAPDH Forward 5' – GGCCTCCAAGGAGTAAGACC (SEQ ID NO: 30) ; GAPDH Reverse 5' – AGGGGTCTACATGGCAACTG (SEQ ID NO: 31);

QSOX1-S Forward 5' – TGGTCTAGCCACAACAGGGTCAAT (SEQ ID NO: 32);
15   QSOX1-S Reverse 5' – TGTGGCAGGCAGAACAAAGTTCAC (SEQ ID NO: 33);
QSOX1-L Forward 5' - TTGCTCCTT GTCTGGCCTAGAAGT (SEQ ID NO: 34);
QSOX1-L Reverse 5' – TGTGTCAAAGGAGCTCTCTGTCCT (SEQ ID NO: 35);

MMP-2 Forward 5' – TTGACGGTAAGGACGGACTC (SEQ ID NO: 36);
20   MMP-2 Reverse 5' – ACTTGCAGTACTCCCCATCG (SEQ ID NO: 37);
MMP-9 Forward 5' – TTGACAGCGACAAGAAGTGG (SEQ ID NO: 38); and
MMP-9 Reverse 5' – CCCTCAGTGAAGCGGTACAT. (SEQ ID NO: 39)   Each reaction was performed in triplicate with the data representing the averages of one experiment.
25   In the shRNA experiment, expression of MMPs was normalized to the non-targeted GAPDH to determine ΔCq. ΔCq replicates were then exponentially transformed to the ΔCq expression after which they were averaged ± standard deviation. The average was then normalized to the expression of the shScramble control to obtain the ΔΔCq expression. Significance was determined using the student
30   two tailed T-test.

Matrigel™ Invasion Assay

Invasion assays were performed using BD BioCoat™ BD Matrigel™ Invasion chambers with 8.0 µm pore size polyethylene terephthalate (PET) membrane inserts

in 24-well format. The assay was performed according to the manufacturers' instructions (BD Bioscience). 4×10^4 cells/well were seeded into the inner Matrigel^{TM} chamber in serum free DMEM. The outer chamber contained 10% FBS in DMEM. BxPC3 and Panc-1 cells were incubated for 24 hours at 37^0C, 5% CO_2. Cells that

5   invaded through the Matrigel^{TM} 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 three wells (6 fields per insert) at 10x, 20x and 40x magnification and the extent of invasion was expressed as the mean +/- S.D. Significance was

10   determined using the Student's two tailed T-test. Results presented are from one of three independent experiments.

RESULTS

Detection of QSOX1 by Immunohistochemistry and Western blot

15   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 two patients, 1016 and 1010 (Fig. 1B, C, and D). 29 of 37 tumor tissues were positive for QSOX1 expression,

20   suggesting it is a commonly over-expressed 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. While our adjacent

25   normal samples indicate a high level of QSOX1 expression, it is hard to determine if there was any tumor tissue contaminating our normal sample, which would account the increase in QSOX1 expression. Western blotting analysis shows that 4 pancreatic tumor cell lines, BxPC3, Panc-1, Capan1 and CFPac1 strongly express QSOX1-S and weakly express the longer splice variant, QSOX1-L. HPDE6, an immortal, non-

30   tumorigenic 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 appears to be a commonly over-expressed protein in PDA (Fig. 1B-C). Second, QSOX1 protein

expression in adjacent normal 1016, 1010, and HPDE6, a non-tumorigenic pancreatic duct cell line, is much weaker than it is in the patient tumor samples and four malignant pancreatic tumor cell lines. This may suggest that QSOX1 provides some advantage to malignant cells that non-malignant 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 demonstrate that the shQSOX1 are active in both cell lines, figure 2A-B shows reduced protein expression of both isoforms of QSOX1 in BxPC3 and Panc-1 tumor cell lines compared to scrambled shRNA in western blot analysis. This experiment demonstrates that sh742, sh528 and sh616 knock down of QSOX1-S expression in BxPC3 cells was 56%, 40% and 28%, respectively; for Panc-1 cells the knock down was 64%, 46% and 18%, respectively (Fig. 2A-B).

ShQSOX1-transduced BxPC3 and Panc-1 cells exhibited a decrease in cell growth compared to shScrambled controls in an MTT assay (Fig. 2C) and by trypan blue viable cell dye (not shown). We seeded an equal number of shScramble, sh742, sh528 and sh616 cells in 96 well plates and quantified the proliferation rate by measuring mitochondrial metabolism on days 1, 2 and 5. While on day 1 there was no change, day 2 presented a minor decrease in cell growth by day 5 BxPC3 sh742, sh528 and sh616 showed a 65%, 60% and 37% decrease, while in Panc-1 sh742, sh528 and sh616 there was a 84%, 88% and 61% decrease. Live cell counts using trypan blue confirmed the MTT assay (not shown).

Cell Cycle and Apoptosis Analysis

Previous work has correlated QSOX1 expression with the quiescent stage, G_o, of the cell cycle (10), leading us to hypothesize if the shQSOX1-mediate 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 to our untreated and scrambled control (data not shown). The results show that the reduced expression of QSOX1 on cell cycle could be cell dependent. BxPC3 showed an increase in G_1 and a significant decrease in S, while Panc-1 cells showed a

5   significant decrease in G_1 but no changes in S.

We further evaluated if 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 to untreated and shScramble a consistent increase

10   of 2-8% 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. Indicating that the reduced expression of QSOX1 does lead to cell death but does not entirely account for the dramatic decrease in cellular proliferation. This data also agrees with our viable cell count revealing a largely nonsignificant decrease in shQSOX1 viable

15   cells compared to untreated and shScramble controls.

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

20   fibronectin before it can migrate into the blood stream and re-establish itself in a distant organ (3). To evaluate whether over-expression of QSOX1 in BxPC3 and/or Panc-1 cells plays a role in metastasis we performed invasion assays over an 18-hour period. Untreated, shScramble, sh742, sh528 and sh616-transduced cells were plated in serum-free medium on Matrigel™-coated, 8um pore inserts. Inserts were placed

25   into wells containing 10% FBS in DMEM. After 18 hours of incubation, tumor cells that had degraded Matrigel™ and migrated through 8um pores onto the underside of the insert were counted (Fig. 4A-B). Our results clearly demonstrate 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

30   nutrient rich media.

Mechanism of Invasion

Since knock-down 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 -9 are key contributors of invasion and metastasis in pancreatic cancer (2). Both, pro-MMP-2 and -9 mRNA and protein levels are elevated in pancreatic tumors, and activated MMP-2 (a-MMP2) appears to be key contributors of metastasis in PDA (2, 22). Because QSOX1 has

5   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 -9 proteins. Untreated BxPC3 and Panc-1 cells, as well as transduced shScramble, sh742, sh528 and sh616 were incubated for 18-24 hours in serum free media after which supernatants were collected and subjected to gelatin-SDS-PAGE. Gelatin

10   zymography was performed to determine if 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 (130kDa), a large amount of proteolytically active pro-MMP-9 (92kDa) with lesser

15   concentrations of pro-MMP-2 (72kDa) and a-MMP-2 (66kDa). Panc-1 supernatants contain less prominent MMP-9 homodimer, pro-MMP-9 (92kDa) and a large amount of proteolytically active pro-MMP-2 (72kDa), unlike BxPC3 cells.

          Supernatants from BxPC3 cells transduced with sh742, sh528 and sh616 showed a 65%, 47% and 10% decrease, respectfully, in pro-MMP9 compared to

20   shScramble (Fig 5A). Supernatants from Panc-1 cells tranduced with sh742, sh528 and sh616 showed a 70%, 56% and 15% decrease, respectfully, in pro-MMP-2. Thus, decreases in the proteolytic activity of MMP-2 and -9, using gelatin as a substrate, provide a mechanism for the shQSOX1-mediated suppression of invasion through Matrigel[TM].

25   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 -9 (Fig. 5C). While our results indicate a slight decrease in MMP-2 and -9 (between 1 and 10% decrease using densitometry analysis) in BxPC3 and Panc-1 shQSOX1 treated cells it is nowhere near the level shown using gelatin zymography. This could be

30   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 post-translationally, we performed quantitative real time PCR (QRTPCR) on MMP-2 and MMP-9 comparing the transcripts from shQSOX1 transduced cell lines with

shScrambled. Figure 5 demonstrates that MMP-2 and -9 RNA increased in the shQSOX1 transduced cells compared to control cells. This result adds confidence to our hypothesis that QSOX1 does not transcriptionally suppress MMP production, rather it post-translationally suppresses MMP activity. 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 five 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 therapeutics that could be used alone or in combination with other anti-neoplastic agents. Prior to our report (4), it was not previously known that QSOX1 was over-expressed in pancreatic tumors. The results presented in figure 1 suggest that QSOX1 is a commonly over-expressed protein in PDA, making it a potential target. To extend those initial findings we began to investigate why pancreatic tumors over-express 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, while the results with our strongest shQSOX1, sh742, show a greater that 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, S2). Contrary to previous statements implicating QSOX1 as a cell cycle regulator (24), our results suggest that while the loss of QSOX1 in Panc-1 cells shows a consistent decrease in G_1, there 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 (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. While our strongest knock-down, sh742, does show at its greatest an 8% increase in annexin V/ propidium iodide 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 protein disulfide isomerase (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. Since suppression of QSOX1 did not appear to play a major role in tumor cell growth, we hypothesized that the over-expression 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[TM] 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 cells lines (27). To determine if this reasoning was correct, we performed gelatin zymography as a way to analyze the matrix metalloproteinase activity.

As a sulfhydryl oxidase, it is unlikely that QSOX1 would directly degrade basement membrane components. 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 figure 5A and B, we found that suppression of QSOX1 leads to a decrease in pro-MMP-2 and -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
5   inhibitors of MMPs (TIMP) 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
10   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 -9 have been found to play an important role in pancreatic cancer progression with 93% of tumors expressing MMP-2 compared to normal tissue (28). While reports implicating MMP-9 in the progression of pancreatic cancer are limited, Tian
15   reported the proteomic identification of MMP-9 in pancreatic juice from patients with pancreatic ductal adenocarcinoma (29). Pryczynicz et al. 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 (2, 22, 28).
20   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
25   substrates. This will be investigated in future work.

Following up on our initial hypothesis regarding MMP activation by QSOX1 we performed a western blot analysis on the same serum free conditioned media that was used to perform gelatin zymography. Our result revealed that the overall levels of secreted MMP-2 and -9 are nearly equal among the untreated, shScramble and
30   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 figure 5A, B and C. To further confirm that what we are observing is a post-translational event we performed qPCR on BxPC3 and Panc-1 shQSOX1 treated cells (Fig. 5D-E). Our observation was

surprising in that we are able to show that there is an overall increase in the transcription of MMP-2 and -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.

5   QSOX1 was previously reported by our group to be over-expressed 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 the present study we demonstrated for the first time that expression of QSOX1 in pancreatic tumor cells directly contributes to an invasive and potentially metastatic phenotype through the

10   activation of MMP-2 and -9 through an as yet undetermined mechanism. It is not known if QSOX1 is solely responsible for the proper folding of MMPs intracellularly, or if it cooperates with protein disulfide isomerase while MMPs are folding in the ER and golgi. Since MMPs are secreted extracellularly where they may undergo autoactivation or cleavage with proteases such as plasmin, it is possible that QSOX1-

15   S activates them in the extracellular environment.

At this point, the post-translational mechanism by which QSOX1 activates MMPs is not clear. Our results indicate that MMP-2 and -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

20   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

25   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.

References for Example 1

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Example 2

We designed two short hairpin RNAs (shRNA1 and shRHA2) to knock-down QSOX1 protein expression in tumor cells.

shRNA1 – Targeting QSOX1 sequence begins at the 970th base pair – ATCTACATGGCTGACCTGGAA (SEQ ID NO: 17)

shRNA2 – Targeting QSOX1 sequence begins at the 1207th base pair – AGGAAAGAGGGTGCCGTTCTT (SEQ ID NO: 18)

QSOX1 shRNA1 hybridizes with nucleotides 970-989 of the QSOX1 transcript. QSOX1 shRNA2 hybridizes to nucleotides 1207-1226 of the transcript. Both shRNAs, independently and together suppressed the production of QSOX1 protein (data not shown) and cell proliferation. Upon cloning the QSOX1 shRNA into a eukaryotic expression vector (pCS2 mammalian overexpression vector with a CMV promoter) and using it to_transfect a pancreatic tumor cell line (BxPC-3), a 30-40% decrease in cell viability was observed, over a 4 to 6 day period, compared to

untreated and scrambled shRNA controls. (**Figure 6**) Using the same transfection

protocol in a separate experiment, we made an additional observation that QSOX1

shRNA-transfected BxPC-3 cells were inhibited from invading through a Matrigel^{TM}

basement membrane composed of collagen, laminin and fibronectin approximately

70% when both QSOX1 shRNAs were combined (**Figure 7**).

A summary of the data obtained from these studies is shown in the table

below:

Sample	% Decrease in QSOX1 protein expression	Invasion of tumor cells through a basement membrane	Viability/mitochondrial respiration
shRNA1	37	63	39
shRNA2	10	69	48
shRNA1/2	41	77	50

Values in table represent % decreases in protein expression or activity

compared to scrambled shRNA in the same CMV driven pCS2 mammalian

overexpression vector., Knockdown is transient in this pCS2 vector.

We claim

1.   A method for tumor treatment, comprising administering to a subject having a tumor an amount effective of an inhibitor of quiescin sulfhydryl oxidase 1 (QSOX1) expression and/or activity, or a pharmaceutically acceptable salt thereof, to treat the tumor.

2.   The method of claim 1, wherein the inhibitor of QSOX1 is selected from the group consisting of anti-QSOX1 antibodies, QSOX1-binding aptamers, QSOX1 antisense oligonucleotides, QSOX1 siRNA, and QSOX1.

3.   The method of claim 1 or 2, wherein the tumor is a tumor that over-expresses QSOX1 compared to control.

4.   The method of any of claims 1-3, wherein the subject is one from which tumor-derived QSOX1 peptides can be obtained.

5.   The method of claim 4, wherein the tumor-derived QSOX1 peptides are selected from the group consisting of NEQEQPLGQWHLS (SEQ ID NO: 3), NEQEQPLGQWH (SEQ ID NO: 4), EQPLGQWHLS (SEQ ID NO: 5), AAPGQEPPEHMAELQR (SEQ ID NO: 6), AAPGQEPPEHMAELQ (SEQ ID NO: 7), AAPGQEPPEHMAELQRNEQEQPLGQWHLS (SEQ ID NO: 8), NEQEQPL (SEQ ID NO: 9), and GQWHLS (SEQ ID NO: 10).

6.   The method of claim 4 or 5 wherein the tumor-derived QSOX1 peptides are obtained from a tissue sample selected from the group consisting of plasma, serum, urine, saliva, and tumor tissue.

7.   The method of any of claims 1-6, wherein the tumor is a pancreatic tumor.

8.   The method of claim 7, wherein the pancreatic tumor comprises a pancreatic adenocarcinoma.

9.   The method of any of claims 1-8, wherein the inhibitor comprises an isolated nucleic acid molecule selected from the group comprising antisense, siRNA, miRNA, and/or shRNA having a nucleic acid sequence perfectly complementary to at least 10 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO: 2, or an RNA equivalent thereof.

10.   The method of claim 9, where the inhibitor comprises a nucleic acid selected from the group consisting of:

5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID NO:11)

5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID
NO:12),

5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID
NO:13),

5   5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

10   5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

15   11.   The method of claim 9, wherein the inhibitor comprises a nucleic acid of the
general formula: CCGG-X1-CTCGAGAAACTTTCTCACCACATTGGC
TTTTTG - 3' (SEQ ID NO: 23)

wherein X1 is a nucleic acid sequence selected from the group consisting of

5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID

20   NO:11)

5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID
NO:12),

5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID
NO:13),

25   5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

30   5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

12.   The method of any of claims 9-11, wherein the nucleic acid inhibitor is administered to the subject in a viral vector.

13.   The method of any one of claims 1-12, wherein the method is for limiting tumor metastasis.

14.   An isolated nucleic acid comprising anantisense, siRNA, miRNA, and/or shRNA molecule having a nucleic acid sequence perfectly complementary to least 10 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:2, or an RNA equivalent thereof.

15.   The isolated nucleic acid of claim 14 comprising a nucleotide sequence selected from the group consisting of

        5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID NO:11)

        5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID NO:12),

        5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID NO:13),

        5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

        5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

        5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

        5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

        5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

        5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

        5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

        5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

        5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

16.   A short hairpin RNA (shRNA) comprising the isolated nucleic acid of claim 15.

17.   The shRNA of claim 16, wherein the shRNA is of the general formula:
CCGG-X1-CTCGAGAAACTTTCTCACCACATTGGC
TTTTTG - 3' (SEQ ID NO: 23)

        wherein X1 is a nucleic acid sequence selected from the group consisting of

        5'-A(T/U)C(T/U)ACA(T/U)GGC(T/U)GACC(T/U)GGAA-3' (SEQ ID NO:11)

5'-AGGAAAGAGGG(T/U)GCCG(T/U)(T/U)C(T/U)(T/U)-3' (SEQ ID NO:12),

5'-GCCAA(T/U)G(T/U)GG(T/U)GAGAAAG(T/U)(T/U)(T/U)-3' (SEQ ID NO:13),

5   5'-GCCAAGAAGG(T/U)GAAC(T/U)GGA(T/U)(T/U)-3' (SEQ ID NO:14).

5'-CCGGACAA(T/U)GAAGAAGCC(T/U)(T/U)(T/U)-3' (SEQ ID NO:15)

5'-(T/U)C(T/U)AGCCACAACAGGG(T/U)CAA(T/U)-3' (SEQ ID NO:16)

5'-ATCTACATGGCTGACCTGGAA-3' (SEQ ID NO:17),

5'-AGGAAAGAGGGTGCCGTTCTT-3' (SEQ ID NO:18),

10   5'-GCCAATGTGGTGAGAAAGTTT-3' (SEQ ID NO:19),

5'- GCCAAGAAGGTGAACTGGATT-3' (SEQ ID NO:20),

5'-CCGGACAATGAAGAAGCCTTT-3' (SEQ ID NO:21); and

5'-TCTAGCCACAACAGGGTCAAT-3' (SEQ ID NO:22).

15   18.   A recombinant expression vector comprising the isolated nucleic acid of any of claim 14-17 operatively linked to a promoter.

19.   The recombinant expression vector of claim 18, wherein the vector comprises a viral vector.

20.   A recombinant host cell comprising the recombinant expression vector of

20   claim 18 or 19.

21.   A pharmaceutical composition, comprising

(a)   the isolated nucleic acid of any of claims 14-17, the recombinant expression vector of claim 18 or 19, or the recombinant host cell of claim 20; and

(b)   a pharmaceutically acceptable carrier.

25   22.   A method for identifying candidate compounds for treating a tumor, comprising

(a)   contacting tumor cells capable of expressing QSOX1 with one or more candidate compounds under conditions suitable for expression of QSOX1;

(b)   determining a level of QSOX1 expression and/or activity in the tumor

30   cells and comparing to control;

wherein a compound that decreases QSOX1 expression and/or activity in the tumor cells relative to control is a candidate compound for treating a tumor.

23.   The method of claim 22 wherein the tumor cells are pancreatic tumor cells.

24. The method of claim 23, wherein the pancreatic tumor cells comprise pancreatic adenocarcinoma cells.

25. The method of any of claims 22-24, wherein the control comprises a tumor cell contacted with the isolated nucleic acid of any of claims 14-17.
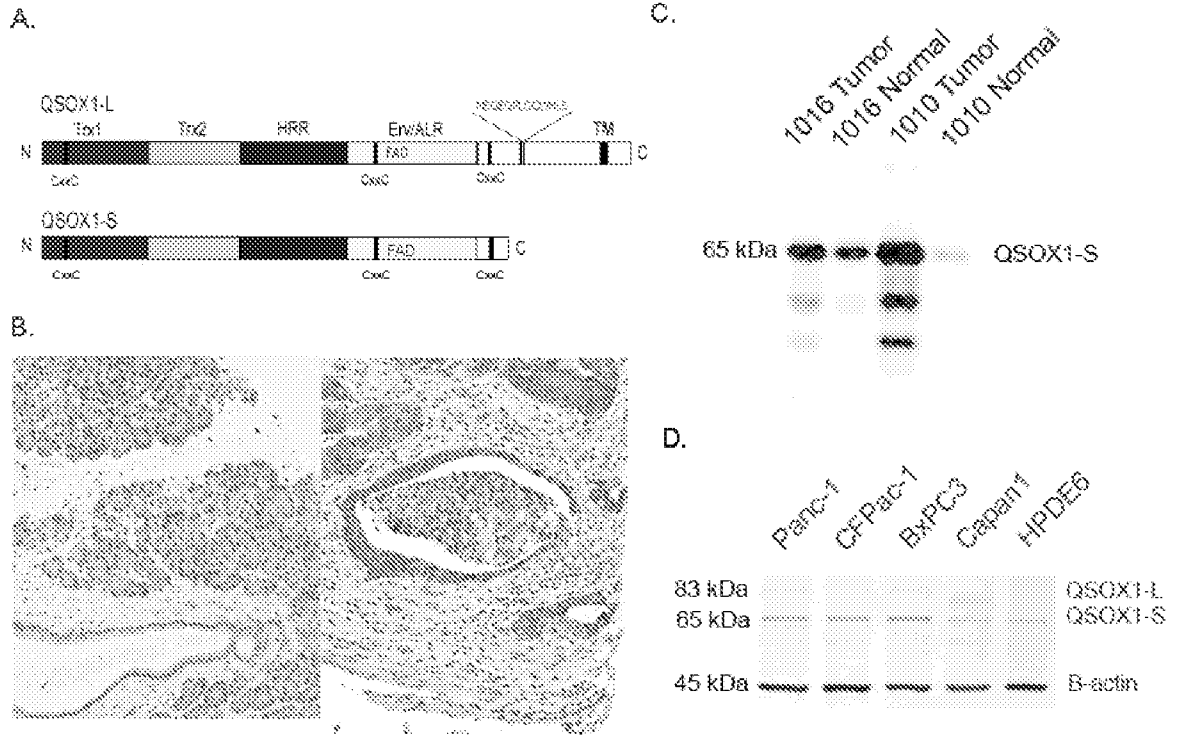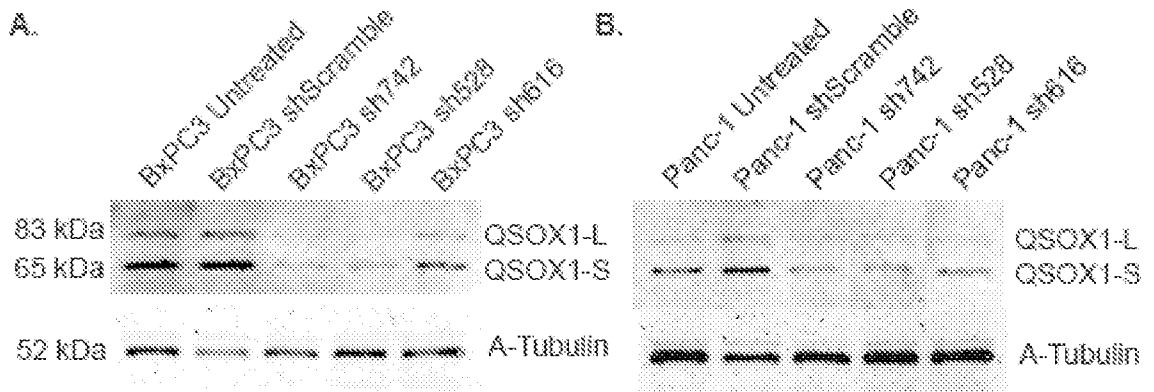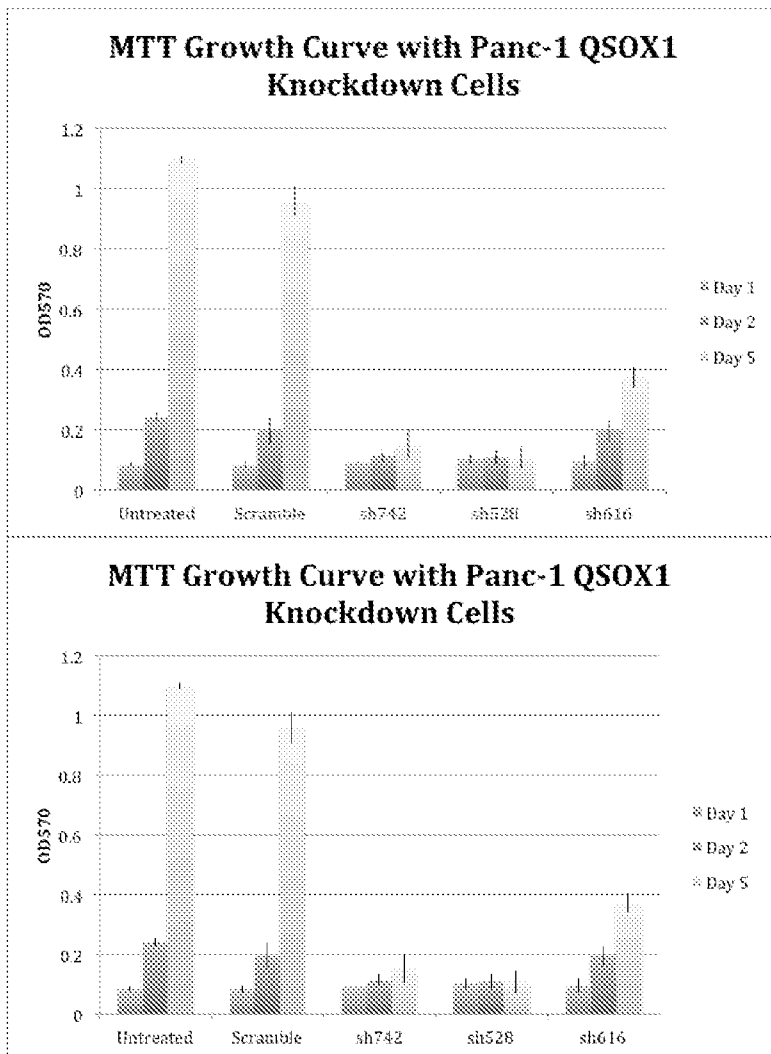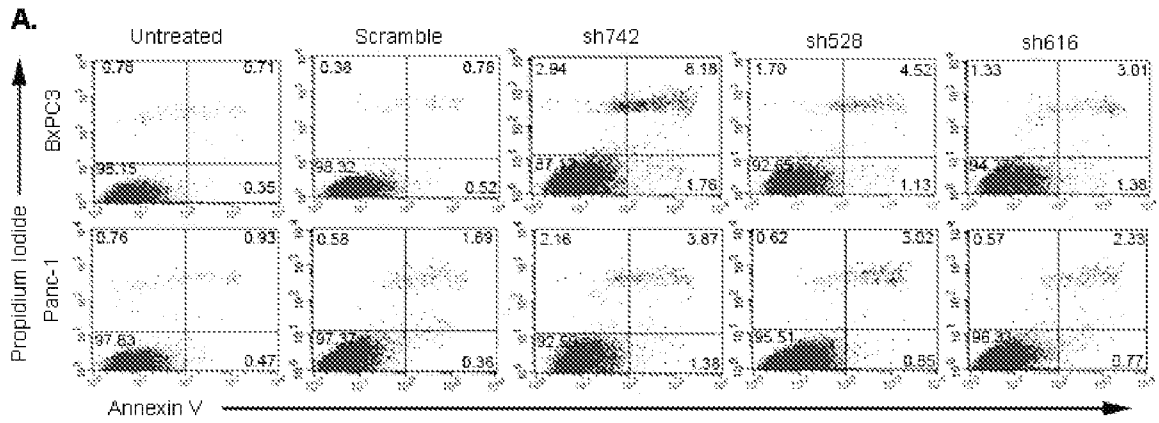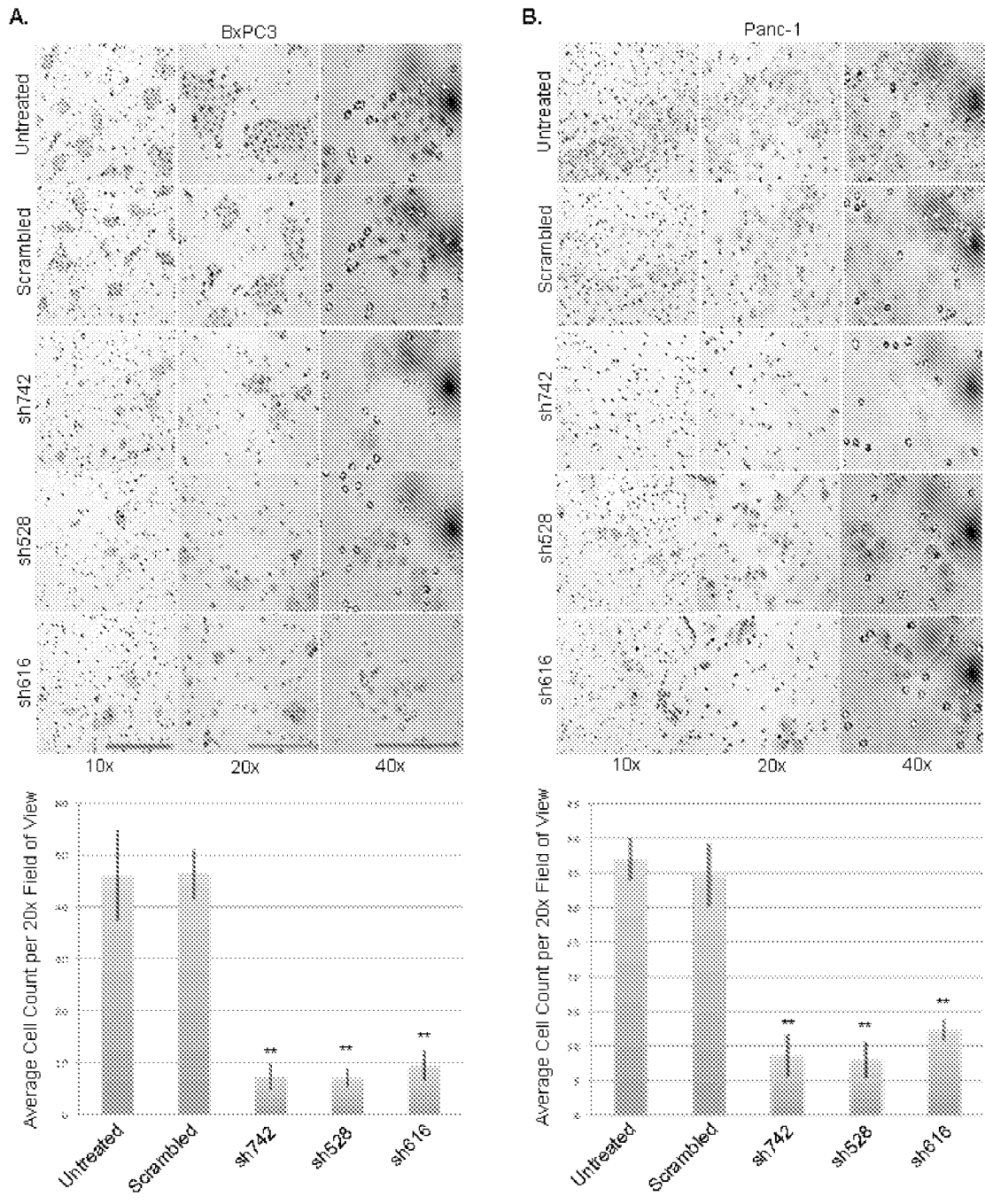
5

Figure 1

Figure 2

Figure 3

Figure 4
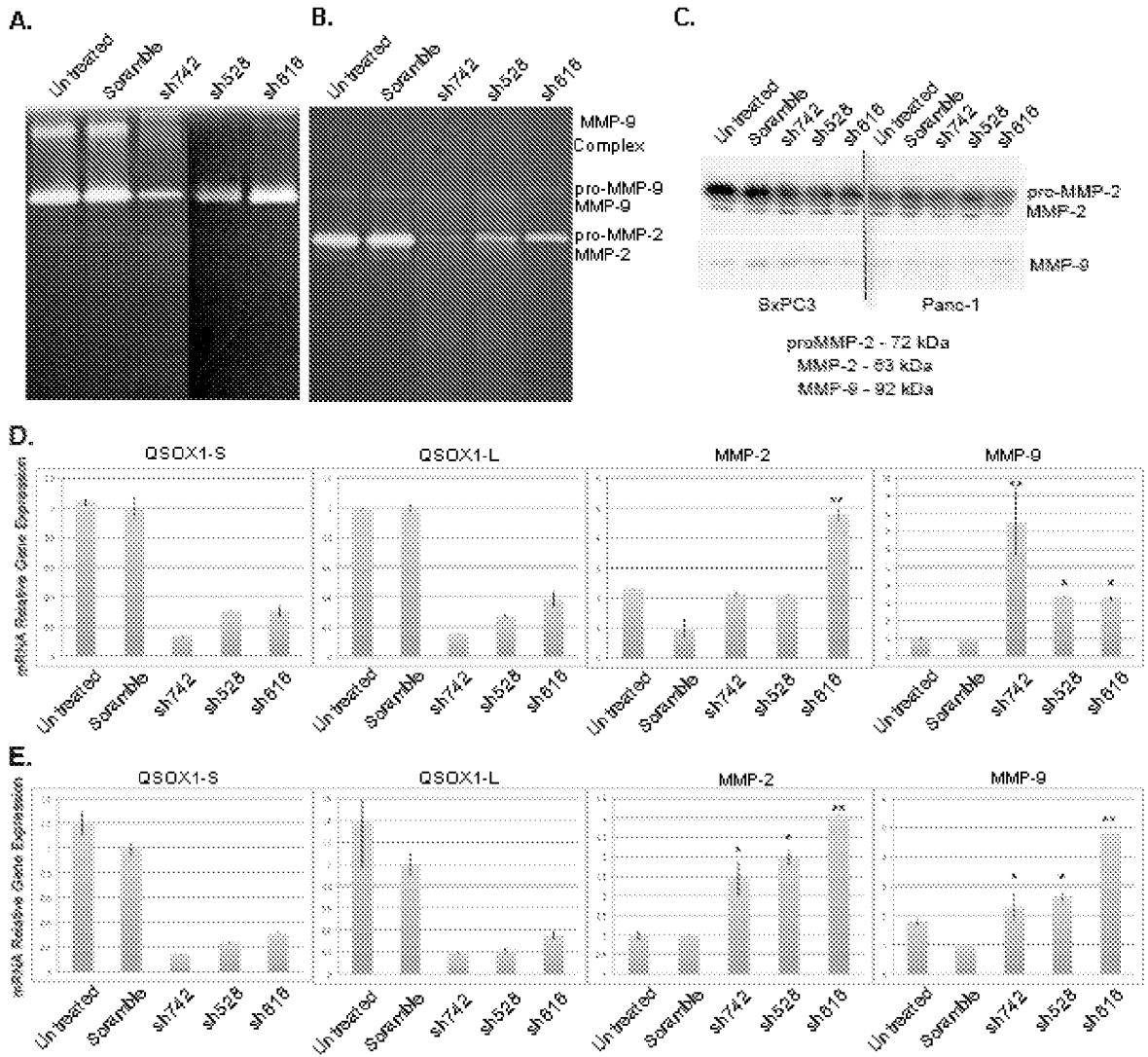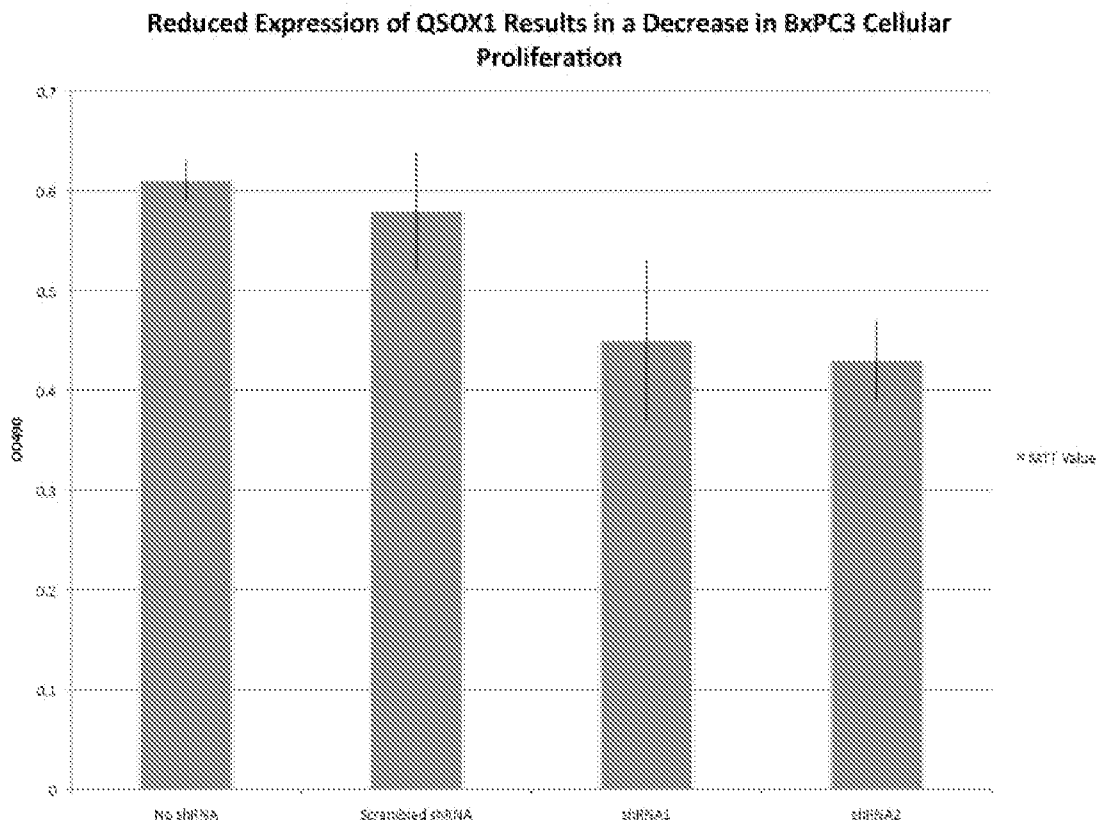
Figure 5

Figure 6

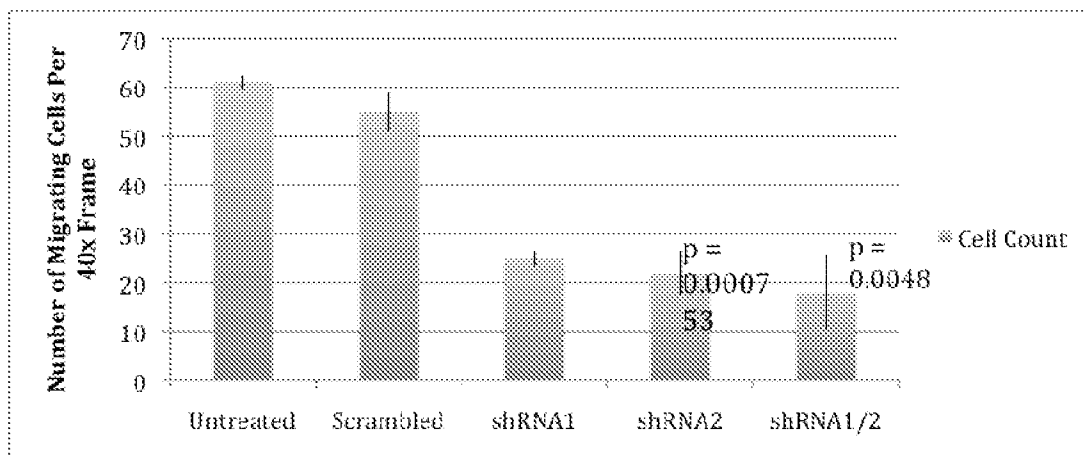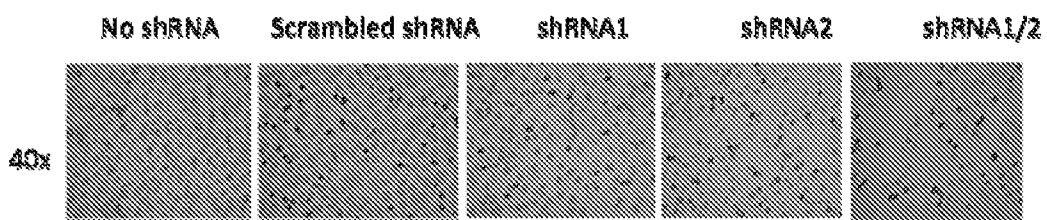Figure 7

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/44   C12N9/02   C12N15/113   A61P35/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OUYANG X ET AL: "Loss-of-function of Nkx3.1 promotes increased oxidative damage in prostate carcinogenesis", CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 65, no. 15, 1 August 2005 (2005-08-01), pages 6773-6779, XP009129575, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-05-1948	1
Y	the whole document	2-13, 22-25

X	WO 2010/086384 A1 (PRONOTA N V [BE]; KAS KOEN [BE]) 5 August 2010 (2010-08-05)	14-21
Y	claims 4,5 page 23, line 21 - page 24, line 6	2-13, 22-25

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[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 2011	29/11/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hars, Jesko

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D. M.E.I. HELLEBREKERS ET AL: "Identification of Epigenetically Silenced Genes in Tumor Endothelial Cells", CANCER RESEARCH, vol. 67, no. 9, 1 May 2007 (2007-05-01), pages 4138-4148, XP55011652, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-06-3032 the whole document & D. M.E.I. Hellebrekers ET AL: "Supplemental Table 4. siRNA sequences", Cancer Research, 4 May 2007 (2007-05-04), pages 1-1, XP55011655, Retrieved from the Internet: URL:http://cancerres.aacrjournals.org/content/suppl/2007/04/26/67.9.4138.DC1/SupplementaryTable_4.pdf [retrieved on 2011-11-09]	14-21
X,P	Jack Geltosky: "QSOX1 as an anti-neoplastic drug target", , 30 August 2011 (2011-08-30), pages 1-1, XP55011559, Retrieved from the Internet: URL:http://azte.technologypublisher.com/files/sites/ncs-m11-003.pdf [retrieved on 2011-11-08] the whole document	1-25
X	ANTWI KWASI ET AL: "Analysis of the plasma peptidome from pancreas cancer patients connects a peptide in plasma to overexpression of the parent protein in tumors", JOURNAL OF PROTEOME RESEARCH, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, US, vol. 8, no. 10, 2 October 2009 (2009-10-02), pages 4722-4731, XP009129574, ISSN: 1535-3907, DOI: 10.1021/PR900414F [retrieved on 2009-08-22] the whole document	1-25

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOREL ET AL: "Involvement of sulfhydryl oxidase QSOX1 in the protection of cells against oxidative stress-induced apoptosis", EXPERIMENTAL CELL RESEARCH, ACADEMIC PRESS, US, vol. 313, no. 19, 26 October 2007 (2007-10-26), pages 3971-3982, XP022317418, ISSN: 0014-4827, DOI: 10.1016/J.YEXCR.2007.09.003 the whole document	1-25
A	WO 2010/071787 A1 (ARIZONA BOARD REGENTS A BODY C [US]; LAKE DOUGLAS F [US]; ANTWI KWASI) 24 June 2010 (2010-06-24) page 9, line 15 - line 29	1-25
A	WO 2010/071788 A1 (UNIV ARIZONA [US]; LAKE DOUGLAS F [US]; RUIZ YVETTE [US]; ANTWI KWASI) 24 June 2010 (2010-06-24) page 9, line 6 - line 34 page 12, line 14 - line 30 claim 17	1-25
A	DATABASE UniProt [Online] 10 August 2010 (2010-08-10), anonymous: "QSOX1_HUMAN", XP002663151, retrieved from www.uniprot.org accession no. 000391 Database accession no. 000391 the whole document	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 2010086384	A1	05-08-2010	NONE	
WO 2010071787	A1	24-06-2010	NONE	
WO 2010071788	A1	24-06-2010	NONE	