From Plasma Peptide to Phenotype: The Emerging Role of Quiescin Sulfhydryl Oxidase 1 in Tumor Cell Biology.

by

Benjamin A. Katchman

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Graduate Supervisory Committee:

Douglas F. Lake, Chair
Jeffery A. Rawls
Laurence J. Miller
Yung Chang

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ABSTRACT

Cancer is a disease that affects millions of people worldwide each year. The metastatic progression of cancer is the number one reason for cancer related deaths. Cancer preventions rely on the early identification of tumor cells as well as a detailed understanding of cancer as a whole. Identifying proteins specific to tumor cells provide an opportunity to develop noninvasive clinical tests and further our understanding of tumor biology. Using liquid chromatography-mass spectrometry (LC-MS/MS) a short peptide was identified in pancreatic cancer patient plasma that was not found in normal samples, and mapped back to QSOX1 protein. Immunohistochemistry was performed probing for QSOX1 in tumor tissue and discovered that QSOX1 is highly over-expressed in pancreatic and breast tumors. QSOX1 is a FAD-dependent sulfhydryl oxidase that is extremely efficient at forming disulfide bonds in nascent proteins. While the enzymology of QSOX1 has been well studied, the tumor biology of QSOX1 has not been studied. To begin to determine the advantage that QSOX1 over-expression provides to tumors, short hairpin RNA (shRNA) were used to reduce the expression of QSOX1 in human tumor cell lines. Following the loss of QSOX1 growth rate, apoptosis, cell cycle and invasive potential were compared between tumor cells transduced with shQSOX1 and control tumor cells. Knock-down of QSOX1 protein suppressed tumor cell growth but had no effect on apoptosis and cell cycle regulation. However, shQSOX1 dramatically inhibited the abilities

both pancreatic and breast tumor cells to invade through Matrigel in a modified Boyden chamber assay. Mechanistically, shQSOX1-transduced tumor cells secreted MMP-2 and -9 that were less active than MMP-2 and -9 from control cells. Taken together, these results suggest that the mechanism of QSOX1-mediated tumor cell invasion is through the post-translational activation of MMPs. This dissertation represents the first in-depth study of the role that QSOX1 plays in tumor cell biology.

DEDICATION

I dedicate this dissertation to my wonderful family. My father Don who has supported me throughout my life, my brother Brad and my sister Brooke who are always there to help me. Especially to my patient and understanding wife Emily for her constant encouragement.

Finally, to my late mother Wendy who always believed in me. You are my biggest inspiration.

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INTRODUCTION

Cancer is heterogeneous disease that, at the most basic levels of detection and treatment, presents researchers and clinicians with a myriad of challenges. While tumor heterogeneity makes it difficult to identify one or two tumor specific biomarkers or therapeutic targets, it certainly provides researchers with exciting opportunities to understand complex genetic and post-translational mechanisms that drive growth and development. Understanding of how these genetic events lead to initiation and progression of cancer are key to the development of prognostic biomarkers and tumor specific inhibitors that can arrest tumor cell growth.

Advances in cancer research in the past 30 years have produced a complex and sometimes overwhelming body of knowledge, detailing characteristics that drive cancer progression. As research continues our understanding of complex networks that drive tumor progression need to be organized into characteristics that are common among tumors. This will lead to the logical progression of new findings in research to anti-neoplastic therapeutics (bench to bedside), where the complexities of the disease can be understood in terms of the underlying principles termed “The Hallmarks of Cancer”. In a seminal review, Douglas Hanahan and Robert A. Weinberg provide a rational for the characterization of the traits that are common to most tumors such as: self sufficiency in growth signals, insensitivity to anti-growth signals, evasion of cellular apoptotic

mechanisms, limitless replicative potential, sustained angiogenesis and tissue invasion and mestastasis (Hanahan and Weinberg). As research continues the hallmarks of cancer will undoubtedly expand into areas of cellular energetics, genome instability, inflammation, evasion of immune destruction mechanisms and the role of the tumor microenvironment in tumor cell proliferation and metastasis (Hanahan and Weinberg). The recent increased efficiency of proteomic and genomic analysis has greatly expanded our discovery of novel proteins and genes expressed during cancer progression. The hallmarks of cancer provide us with a starting point at which to evaluate how over-expression of one gene or protein plays a role in tumor development.

Autonomous Proliferative Signaling

Arguably, one of the most fundamental traits of a dysplastic cell is growth autonomy. Normal cells rely on autocrine or paracrine mitogenic signals to initiate growth. Transmembrane receptors such as receptor tyrosine kinases interact with growth factors initiating a cascade of events that drive cellular growth (Witsch, Sela, and Yarden). Epithelial cells rely on numerous integrins that interact with basement membrane components and extracellular matrix proteins to initiate, as well as terminate, proliferation (Lemmon and Schlessinger).

In contrast, tumor cells show a reduced dependence on external mitogenic factors to proliferate, often generating their own stimulatory

signals decreasing their dependence on growth factors. Proliferative autonomy disrupts key homeostatic mechanisms that ensure controlled cell growth, death and tissue development and maintenance. There are three common strategies that tumor cells utilize to achieve growth autonomy: alterations in external growth signals, hypersensitive cell membrane transducers of those signals, and constitutive expression of intracellular signals that translate growth signals into uncontrolled cellular proliferation (Witsch, Sela, and Yarden).

The majority of normal cells receive growth signals in a paracrine manner from neighboring cells. Tumor cells are able to create autocrine signals to support their growth. This eliminates their dependence on neighboring cells to proliferate. Although tumor-stroma interaction have recently been shown to play a role in growth and drug resistance (Place, Huh, and Polyak; Zhang and Huang). New cell culture techniques are only recently allowing us to fully appreciate the role of accessory cells and extracellular components on tumor cell growth. Undoubtedly as this area of research expands it will further shape how we view growth automomy. Platelet derived growth factor (PDGF) is an example of a protein that is normally generated and secreted by stromal cells (Compagni and Christofori). In gliobastomas and sarcomas PDGF is produced independently by the tumor cell and has been shown to drive tumor cell proliferation (Compagni and Christofori).

Research over the past 10 years has seen minimal advances in understanding the identities and sources of these proliferative signals. This is in part due to the current methods used to analyze tumor cell behavior. The majority of research on tumor cells is performed in 2-D monocultures, which does not take into account cell number, position, neighboring cells of different origin and the compounding proteases and other enzymes that sequester and release growth factors from the pericellular space. However, autocrine mechanisms of sustained proliferative tumor cell signaling is better understood than paracrine (Lemmon and Schlessinger; Witsch, Sela, and Yarden). Tumor cells have developed the ability to generate their own growth factor ligands. In conjunction with the overexpression of growth factor ligands, tumor cells may also overexpress cell surface receptors for those ligands. This renders the tumor cell hyperreseponsive to otherwise limiting amounts of growth factors (Witsch, Sela, and Yarden; Cheng et al.).

Transmembrane receptors such as epidermal growth factor-receptor (EGF-R) transduce growth stimulatory signals through receptor tyrosine kinase activity (Lemmon and Schlessinger; Wheeler, Dunn, and Harari). In breast tumors, members of the EGF family (EGF-R/erbB and Her2/neu) are responsible for uncontrolled proliferation and ligand-independent growth through the mitogen activated protein (MAP)-kinase pathway. Overexpression of EGF-R propagates growth autonomy through signal transduction in the cytoplasm that influences cellular behavior:

proliferation, metastasis, evasion of apoptotic mechanisms and cell cycle regulation (Witsch, Sela, and Yarden; Wheeler, Dunn, and Harari).

Increased efficiency of high-throughput and next generation DNA sequencing has led to the identification of somatic mutations in receptor tyrosine kinases and their downstream mitogenic pathways. In melanoma approximately 40% of tumors express mutations in B-Raf resulting in the constitutive signaling of the downstream MAP kinase pathway (Davies and Samuels). Targeting of these growth receptors specifically B-Raf and their downstream pathways has led to tremendous responses in treatment of patients with melanoma. Short interfering RNA (siRNA) and small molecule targeting of B-Raf in patients with advanced melanoma results in immediate regression, within 14 days, of the initial treatment (Flaherty et al.). This not only underscores the advantages that receptor tyrosine kinase pathways provide to tumor but also demonstrates how the inhibition of constitutive growth signals can restore negative feedback mechanisms that normally ensure proper growth regulation.

Circumvention of Anti-Growth Signaling

Phenotypically connected to growth autonomy is the ability of tumor cells to circumvent anti-proliferative signaling. Anti-proliferative signaling is designed to maintain cellular quiescence and tissue homeostasis. Functionally anti-proliferative signals act in two ways to maintain tissue homeostasis; through soluble growth factor inhibitors or

through cell surface receptors either on the cell surface or that of neighboring cells thus forcing cells into a quiescent state (G_0) (WEINBERG). For tumor cells to thrive they must overcome these anti-proliferative signals. The majority of these proteins are associated with cell cycle, specifically the transition from G_1 to S phase (Williams and Stoeber).

Cancer researchers over the past 30 years have validated dozens of tumor suppressors through gain- or loss-of-function studies. Two of the most well characterized tumor suppressor proteins are Retinoblastoma-associated protein (RB) and TP53 (Lipinski and Jacks; Ghebranious and Donehower). Both RB and TP53 are key molecules that regulate cell fate decisions (Lipinski and Jacks; Ghebranious and Donehower).

The RB protein integrates multiple signals from external and internal cues that dictate the ability of a cell to proliferate. The absence to RB function by mutation or deletion leads to unchecked cell cycle progression by multiple mechanisms (WEINBERG). In contrast to RB, TP53 mainly functions through internal cues such as stress and cellular abnormality sensors relating to excessive genome damage or suboptimal oxygenation (Ghebranious and Donehower). TP53 can then act to halt cell cycle progression until normal conditions are restored. If there is a failure to restore the cell to normal levels TP53 ultimately will trigger apoptosis. Although the majority of tumors posses mutations in either RB and/or TP53 it is unquestionable that these two proteins function only in the

context of a larger network. RB and TP53 null mice have been shown to develop normally and are surprisingly free of proliferative abnormalities until late in their lives (Lipinski and Jacks; Ghebranious and Donehower). These results reflect redundant signaling mechanisms within the cell. Emphasizing that tumor cells are the result of multiple mutations within redundant pathways ultimately leading to cancer.

Elusion of Cell Death

Overwhelming evidence supported by *in vivo* and *in vitro* models demonstrates that in addition to acquired proliferative abilities all tumor cells develop the ability to evade cellular apoptotic, autophagic and necrotic pathways (Saikumar et al.; Zörnig et al.). The most well characterized method of cell death is apoptosis. Apoptosis is a genetically predetermined mechanism for programmed cell death. Cellular conditions regulating apoptosis are monitored through external (extrinsic) and internal (intrinsic) pathways (Saikumar et al.). The extrinsic pathway is triggered by ligand-induced activation of death receptors at the cell surface (Saikumar et al.). The intrinsic pathway is the result of intracellular cascade of events leading to mitochondrial membrane permeabilization (Saikumar et al.). Each apoptotic pathway can be divided into three phases based on caspase activation: initiation, integration/decision, and execution/degredation (Saikumar et al.). Both pathways ultimately lead to

cell shrinkage, chromatin condensation, mitochondrial membrane disruption, and nuclear fragmentation.

Extrinsic apoptosis involves ligand activation of death receptors and/or dependency receptors on the cell surface (Saikumar et al.; Zörnig et al.). Activation of death receptors, tumor necrosis factor (TNF) family members, causes the Fas-associated death domain-containing protein (FADD) to associate with and oligomerize with the death inducing signaling complex (DISC). DISC then binds the initiator caspases 8 and 10, leading to dimerization and activation of effector/executioner caspases 3, 6, and 7. Rapid caspase activation coming from dependency receptors (e.g. netrin-1), leads to direct activation of effector/executioner caspases (Plati, Bucur, and Khosravi-Far).

The majority of cell death in vertebrates proceeds through the intrinsic apoptotic pathway (Zörnig et al.). The intrinsic pathway is initiated due to DNA damage (nuclear activation). Caspase-2 migrates to the mitochondria causing membrane permeabilization allowing proteins from the inner membrane space (IMS) to be released into the cytosol (Garrido et al.). The intrinsic pathway is also activated by ER stress that releases calcium (Ca^{2+}) into the mitochondria causing cytochrome c release and outer membrane (OM) permeabilization (Garrido et al.). Once the IMS proteins enter the cytosol they follow three distinct paths to induce apoptosis 1.) cytochrome c promotes the activation of the “apoptosome” complex by recruiting the initiator caspase-9 and apoptosis protease

activating factor-1 (APAF-1) as well as ATP/dATP. Activated caspase-9 then catalyzes the activation of effector caspases 3, 6, and 7. 2.) Caspase-independent apoptosis occurs through apoptosis-inducing factor (AIF) and endonuclease G (EndoG). These proteins translocate into the nucleus initiating DNA fragmentation and chromatin condensation. 3.) Lastly, second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO) and Omi stress-regulated endoprotease/high temperature requirement protein A2 (Omi/HtrA2), initiate apoptosis indirectly by binding and sequestering inhibitor of apoptosis protein (IAP) allowing effector caspase activation (Garrido et al.).

Cross talk between the extrinisic and intrinsic pathways has been extensively studied. The main link between the extrinsic and intrinsic pathways is initiated through the proteolytic cleavage of Bid. Bid, a member of the BCL-2 homology domain (BH3) family and is known to interact with members of the Bcl-2 family (Bax, Bak, Bcl2 and Bcl-XL) through the BH3 domain. Full length Bid (26 kDa) is cleaved by caspase-8 into 11 and 15 kDa products. The 15 kDa (tBid) fragment contains the functional BH3 domain that can still interact with member of the Bcl-2 family. There are two ways in which tBid can activate mitochondrial apoptosis: 1) tBid can directly interact with the OM, causing pore formation and the subsequent release of cytochrome c. 2) tBid can interact with the anti-apoptosis Bcl-XL through its BH3 domain preventing the formation of

the anti-apoptosis complex. This allows Bax activation and pore formation in the OM. (Saikumar et al.; Zörnig et al.; Plati, Bucur, and Khosravi-Far)

Tumor cells have developed multiple ways to prevent the release of cytochrome C. Tumor cells have been shown to overexpress Bcl-2 (BH3-only) family members or disrupt the FAS ligand signaling circuit (Plati, Bucur, and Khosravi-Far). One of the most commonly occurring traits is the loss or mutation of tumor suppressor proteins such as TP53. TP53 induces apoptosis due to DNA damage through the upregulation of BH3-only proteins, Noxa and Puma (Saikumar et al.). The loss of TP53 function eliminates this DNA damage sensor from the apoptotic circuitry. Tumors may also overexpress anti-apoptotic regulators (Bcl-2, Bcl-X_L) or survival proteins Insulin growth factor 1/2 (Igf-1/2) through the downregulation of proapoptotic factors (Bax, Bim and Puma) (Zörnig et al.). To circumvent the extrinsic apoptotic pathway tumor cells have been shown to either short circuit the Fas receptor or present a decoy receptor (Plati, Bucur, and Khosravi-Far). The redundancy at which tumor cells evade cellular apoptosis directly reflects the diversity of apoptosis signaling mechanisms that tumor cells encounter as they evolve to a malignant state.

Autophagy represents an alternative to apoptosis in which cells under stress (e.g. nutrient deficiency) will break down cellular organelles. This allows the cellular catabolites to be recycled and used for biosynthesis and energy metabolism (Kundu and C. B. Thompson). Intracellular vesicles termed autophagosomes envelop organelles

subsequently fusing with lysosomes where degradation occurs. The low molecular weight metabolites that are generated are used to support the cell in nutrient deprived environments similar to the environment created by tumor formation.

Numerous pathways have been uncovered detailing the crosstalk between autophagy and apoptosis. Similar proteins that inhibit apoptosis (PI3 kinase, AKT and mTOR kinase) also work to prevent autophagy (Kundu and C. B. Thompson). One essential protein important for autophagosome formation is Beclin-1. Beclin-1 is a member of the BH3-only subfamily of apoptotic regulatory proteins. The BH3 domain allows it to bind to Bcl-2 proteins. Under cellular stress Beclin-1 is displaced from Bcl-2 triggering autophagy (Kundu and C. B. Thompson).

Research into autophagy has revealed that tumor cells utilize the autophagy pathway to survive in a nutrient depleted environment. Paradoxically, autophagy levels are induced in patients being treated with radiotherapy and chemotherapy, impairing rather than enhancing the killing of tumor cells (Hanahan and Weinberg; Kundu and C. B. Thompson). While the role of autophagy in tumor cells is just now emerging, future research will clarify the genetic and physiological conditions that lead to autophagosome formation and how autophagy enables cancer cells to survive or how it leads to cell death.

Necrosis in contrast to apoptosis and autophagy causes a cell to expand and explode, releasing their cellular contents into the tumor

microenvironment (MD). Previously, the role of necrosis in tumor cells was viewed solely in the terms of cell death. Recent research indicates that necrosis is under genetic control under certain circumstances rather than a random event (Hanahan and Weinberg). A consequence of necrosis is the release of inflammatory and pro-growth (IL-1α) signals to neighboring cells (MD; Zörnig et al.). These signals have been shown to recruit tumor promoting immune inflammatory cells, as well as contributing to angiogenesis, proliferation and invasiveness (MD; Kundu and C. B. Thompson). The precise role of necrosis in promoting tumor proliferation is still under study, but chronic inflammation has been shown to drive tumor growth. It is clear that while necrosis appears to be a counter-balance to tumor formation, it may actually promote tumor expansion.

Boundless Replicative Immortality

The ability of tumor cells to develop growth autonomy and to evade cellular apoptotic mechanisms, detaching itself from normal regulatory mechanisms is not enough to acquire limitless replicative potential. Early work by Hayflick and colleagues in cell culture models demonstrated that normal cells have a finite passage number (Hayflick). Once cell populations reach their finite doubling time they enter into senescence or a quiescent state.

Work *in vivo* and *in vitro* has demonstrated two main mechanisms for tumor cells to overcome a senescent state. Tumor cells have been

shown to either disable RB and TP53 tumor suppressors or increase the expression of telomerase allowing these cells to evade cellular senescence and continue to replicate (Artandi and DePinho). As the cells begin to reach their finite passage number (average 60-70 for normal human cells) they enter a crisis state characterized by massive cell death (Artandi and DePinho). Wright and colleagues demonstrated that approximately 1 in 10^7 cells emerge from this crisis state with the ability to replicate without limit (Hayflick). Numerically, 60-70 doublings by tumor cells should be enough to exceed the number of cells in the human body. Two conclusions can be made from ongoing research into senescence; first that cellular senescence is emerging as a protective barrier to neoplastic expansion and second that the number of cells in a tumor does not represent the total amount of tumor cells needed to produce a malignancy.

Angiogenesis

Oxygen and nutrients provided by the vasculature are essential to normal growth and development. The process of angiogenesis addresses this need for normal cells and tumor cells to excrete their waste and take up fresh oxygen and nutrients. During development of the vasculature endothelial cells assemble into tubes (vasculogenesis) leading to the branching of new vessels (angiogenesis) from the existing vascular structure (Lin et al.). After embryological and mature development,

expansion of the vasculature remains largely quiescent (Baeriswyl and Christofori). The exceptions are angiogenesis after injury, during wound healing and female reproduction (Baeriswyl and Christofori). In contrast, tumor expansion is dependent on the development of angiogenesis that brings a blood supply to the tumor (Weis and Cheresh).

As tumors increase in mass an “angiogenic switch” takes place leading to the constitutive expression of pro-angiogenic factors and the downregulation of anti-angiogenic factors (Hanahan and Folkman). The angiogenic switch is regulated by soluble factors and their cell surface receptors (integrins). In addition to cell surface adhesion molecules that interact with neighboring cells and basement membrane proteins, proteases cleave extracellular matrix proteins and cell surface receptors producing pro-angiogenic peptides (Baeriswyl and Christofori).

Vascular endothelial growth factor (VEGF) is the most common angiogenic stimulatory molecule produced during tumorigenesis. The VEGF gene encodes three tyrosine kinase receptors (VEGFR-1-3) (Hanahan and Weinberg; Weis and Cheresh). VEGFR are involved in the generation of new blood vessels from embryonic development through the physiological and pathological circumstances in adults (Lieu et al.). VEGF signaling is regulated and induced on multiple levels either during hypoxic or nutrient deprived situations as well as by oncogenic signals (Cross and Claesson-Welsh; Lieu et al.). VEGF in its latent form can also be released

and activated by matrix metalloproteases (MMP) leading to sustained tumor angiogenesis (Lieu et al.).

In contrast there are numerous anti-angiogenic factors that work to tightly regulate aberrant angiogenic signaling. Thrombospondin-1 (TSP-1) as well as angiostatin are two well-known examples of proteins that counterbalance the angiogenic switch (Taraboletti et al.; Bauvois). They both work by binding to transmembrane receptors on endothelial cells inducing a suppressive signal to counteract the pro-angiogenic signals (Taraboletti et al.). Research over the past 30 years has led to the identification of numerous endogenously produced angiogenic inhibitors (Weis and Cheresh; Baeriswyl and Christofori; Keleg et al.). Transgenic mouse studies have shown that the loss of genes that encode these inhibitors by themselves does not result in autonomous growth but rather enhances the growth of implanted tumors (Weis and Cheresh). If the concentration of the circulating angiogenic inhibitors is increased, tumor growth is impaired as well as wound healing abilities (Weis and Cheresh). Current research suggests that endogenous angiogenesis inhibitors under normal circumstance help to control transient angiogenic capabilities during wound healing and that they also act to control angiogenesis in persistent tumors.

Tumor Cell Invasion and Metastasis

The ability of primary tumor cells to degrade the basement membrane components, invade into adjacent tissue or circulate in blood leading to a secondary metastatic site of tumor growth are the cause of 90% of human cancer deaths (Hanahan and Weinberg). Invasion and metastasis provide tumors with the ability to escape their nutrient depleted environment and colonize nutrient rich areas of the body. As tumor cells progress to a higher pathological grade there is a clear shift in cellular morphology that is reflected at the transcriptional and translational level. While the specific processes of invasion and metastasis are still poorly understood, recent advances in experimental models have broadened our overall context of the metastatic cascade.

The process of invasion and metastasis is performed as discrete steps involving cellular and metabolic changes. As a tumor cell progresses toward a metastatic phenotype, it undergoes a morphological change from a cuboidal shape (epithelial) to a more elongated (mesenchymal) shape (Bacac and Stamenkovic). There is a succession of events starting with the local invasion of tumor cells into neighboring blood vessels (intravasation) followed by the migration of the tumor cells through the lymphatic and hematogenous system (Talmadge and Fidler). The tumor then escapes the vessels into distant tissues (extravasation) where it recolonizes (colonization) as micrometastatic lesions eventually growing into macroscopic tumors (Talmadge; Bacac and Stamenkovic).

The developmental process of epithelial-to-mesenchymal transition (EMT) has now become widely accepted as one of the primary means in which tumor cells acquire the ability to invade and disseminate into local and distant tissue (Bacac and Stamenkovic; Kokkinos et al.). Tumor cells can shift to a mesenchymal state either stably or transiently during the process of metastasis (Sarrio et al.), where upon extravasation plasticity in the EMT process allows the reversal back to an epithelial state resulting in the formation of a new tumor colony. EMT in tumor cells is characterized by a “cadherin-switch” where E-cadherin is replaced by N-cadherin driving growth and invasion (Blick, Widodo, Hugo, Waltham, Lenburg, Neve, and E. W. Thompson; Royer and Lu; Katz et al.). E-cadherin, a cell-to-cell adhesion molecule is one of the best-characterized alterations in invading tumor cells (Solanas et al.). In normal cells E-cadherin regulates growth and invasion through its transmembrane receptor, forming adherent junctions with neighboring cells and basement membrane proteins (Zeisberg and Neilson). The binding of E-cadherin with neighboring cells leads to an increase in anti-growth signals via its cytoplasmic interaction with β-catenin (Solanas et al.). Loss of E-cadherin in the majority of tumor cells either by proteolytic cleavage (metalloproteases and cysteine cathepsin proteases) of the extracellular domain or through transcriptional repression (Snail, Slug, Twist and Zeb1/2) result in the release of β-catenin into the nucleus up-regulating pro-growth signals (Royer and Lu; Moreno-Bueno et al.; E. S. Radisky and D. C. Radisky).

A decrease in E-cadherin is accompanied by an increase in N-cadherin, a protein that is normally associated with migrating neurons and mesenchymal cells during development (Kalluri and Weinberg). A structural shift is also a hallmark of EMT documented by the transition of interfilament proteins, such as keratin to the more invasive filament, vimentin (Kalluri and Weinberg). While it is widely accepted that EMT plays a large role in tumor cell metastasis the extent to which individual components such as transcription factors, cadherins and structural proteins initiate and drive tumor cell metastasis is still under investigation.

Although the expression of EMT promoting transcription factors as well as the structural changes associated with EMT have been observed in the majority to epithelial tumors, it is not the only mechanism at which tumor cells develop invasive potential. The contributions of the tumor microenvironment provide tumor cells with proteases and pro-invasive factors that alleviate the need for tumor cells to independently generate invasive proteins. MMPs represent the most studied proteases that contribute to enhanced growth and metastasis in tumor cells.

MMPs are a family of 28 conserved zinc-associated proteinases that are classified into six groups based on sequence homology as well the substrates in which they primarily degrade (Tallant, Marrero, and Gomis-Rüth). Most MMPs are thought to be secreted in an inactive form from either stromal cells or epithelial cells and then activated in the extracellular environment (Kessenbrock, Plaks, and Werb). Activation of

MMPs occurs through a process called “cysteine switch” in which Cys^{73}-S-Zn in the latent MMP is a site of cleavage, exposing a zinc ion. Further folding of the MMP after cleavage of the pro-domain activate the catalytic domain (Kessenbrock, Plaks, and Werb).

The activity of MMPs is regulated by several types of inhibitors, but the majority of the work is done by tissue inhibitors of MMP (TIMP) (Snoek-van Beurden and den Hoff; Kessenbrock, Plaks, and Werb). The TIMPs are also secreted proteins but can be found bound to the surface of cells in tight association with MT-MMP (Snoek-van Beurden and den Hoff; Kessenbrock, Plaks, and Werb). The balance between the MMPs and TIMPs is what is largely responsible for regulating degredation of the extracellular matrix proteins (Kessenbrock, Plaks, and Werb). Deregulation of the MMPs is a characteristic of numerous pathological conditions including tumor cell invasion (Köhrmann et al.; Zeisberg and Neilson).

The extent and mechanism to which these processes of invasion contribute to metastasis are currently poorly understood. As metastatic models continue to evolve it is undeniable that all of these process described above will interconnect primary tumor invasion and metastasis in multiple ways.

Therapeutic Strategies

An understanding of how the hallmarks of cancer are intertwined and contribute to cancer progression has led to tremendous advances in targeted cancer therapeutics. While my thesis does not yet describe the use of quiescin sulfhydryl oxidase 1 (QSOX1) as a viable target for cancer therapy I feel that it is important to briefly discuss how a molecular understanding of the required traits for cancer development have led, in one example, to success and failure in cancer treatment. The recent development of monoclonal antibody and small molecule inhibitors directed towards a specific receptor (receptor tyrosine kinases) or protein resulting in successful regression of primary tumors has validated the importance of particular hallmark capabilities (Lemmon and Schlessinger). At the same time we have also seen that specific molecular targeting while leading to fewer off-target effects often times has a transitory response eventually followed by a relapse (Lemmon and Schlessinger; Hanahan and Weinberg). The previously described clinical trial involving siRNA targeting of B-Raf in melanoma patients exemplifies this point (Flaherty et al.). Initial treatment of advanced melanoma patients using B-Raf inhibitors leads to an immediate remission in 90% of patients within 14 days of treatment. While treatment of melanoma with B-Raf inhibitors is initially successful almost all patients experience a relapse within two years of their initial treatment (Flaherty et al.). Follow up research into patients that relapsed found that the tumor cells had adapted to the

treatment, utilizing an alternative pathway to drive proliferation through MAPK, a key growth factor in a tumor cells ability to develop growth autonomy (Flaherty et al.). The results of this clinical trial while extremely impressive emphasize the ability of a tumor cell to adapt to specific treatments utilizing redundant pathways. As we continue to mine for cancer specific biomarkers using advances in genomic and proteomic technologies our understanding of tumor promoting pathways will most likely lead to the progression of the combinational targeting of multiple biochemical pathways. A comprehensive understanding of the web of pathways will allow researchers to target the same pathway in multiple ways completely alleviating the tumor burden.

Plasma Biomarker Discovery

The ability to develop clinical tests that can detect cancer at its early stages is equally dependent on the discovery of novel biomarkers as it is on understanding the phenotypes that enable and drive tumor progression. As previously discussed hallmark traits such as growth autonomy, angiogenesis and metastasis are often driven by short peptides cleaved from the cell surface and basement membrane components as well as whole proteins secreted by the local tumor microenvironment and the primary tumor. Because blood circulates through every organ of the body, sampling normal and tumor proteins, it provides a logical source to mine for tumor specific biomarkers.

Cancer is generally a disease of probability in which the earlier one is able to detect disease the higher the success rate in curing the disease with surgery and/or other therapies. Early stage tumors may not have metastasized, for example. Mining for biomarkers in patient plasma is very difficult due to the complexity of highly abundant proteins which obscure less abundant potential biomarkers that could give unique insight into normal versus disease. By filtering out the more abundant plasma proteins in cancer patient samples we were able to find several peptide fragments that were not present in control plasma. We then used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the peptides. Our initial screen identified a unique peptide (NEQEQPLGQWHLS) that was consistently found in patient samples but not in normal control samples. By performing a BLAST search we were able to map this short peptide back to the parent protein QSOX1-L. The NEQEQPLGQWHLS peptide was found in 16 of 23 ductal adenocarcinoma patients (DAP), 4 of 5 patients with intraductal papillary mucinous neoplasm (IPMN), thought to be a precursor to pancreatic ductal adenocarcinoma (PDA) but this peptide was not found in any of the 42 normal healthy donor samples using the same method (Figure 1).

We then performed immunohistochemistry (IHC) on paraffin embedded sections from patients who underwent surgical resection for PDA (Figure 1). We were able to show using a polyclonal antibody to QSOX1 that staining with QSOX1 is very specific to dysplastic cells within

the pancreatic duct and not expressed in adjacent normal cells (Figure 1). QSOX1 protein is not only expressed in the pancreas but it is also weakly expressed in normal transformed cell lines as well as being highly expressed in cancer cell lines such as breast, prostate, colon, brain, and lung. We have also found through western blot analysis of total cell lysate that peripheral blood mononuclear cells (PBMC) taken from a healthy individual do not express QSOX1 but when you treat PBMC's with the mitogen, phytohemagglutin (PHA), QSOX1 becomes weakly expressed. Initially concluding that QSOX1 is activated through cellular stress, possibly as a mechanism to control cellular proliferation. QSOX1 is a protein that has been shown to be expressed at a very low level if at all in normal tissue but is expressed either due to reactive oxygen species in the cell or mitogen stimulation and is highly expressed in a variety of cancers with extremely specific staining in the dysplastic cancer cells (Antwi, Hostetter, Demeure, Katchman, Decker, Ruiz, Sielaff, Koep, and Lake).

QSOX1 protein was initially shown by our lab to be overexpressed in rapidly proliferating cells (tumor or mitogen stimulated cells), promoting cellular survival through an unknown mechanism (Antwi, Hostetter, Demeure, Katchman, Decker, Ruiz, Sielaff, Koep, and Lake; Morel et al.). Tumors arise from the inability of a cell to control proliferation as well as initiate apoptosis. Therapeutic intervention is often thwarted by the overexpression of proteins in response to anti-neoplastic agents that

suppress apoptosis and activate cellular growth cascades that allow the primary tumors to thrive. By determining the role of QSOX1 in response to cellular homeostasis and cancer progression we hope to be able to use QSOX1 as a marker to indicate disease progression or potentially as a tool for therapeutic intervention in combination with current therapeutic regimens. Thus beginning our investigation on the function of the sulfhydryl oxidase QSOX1 as it relates to the above described hallmark of cancer traits.

Sulfhydryl Oxidase Family

Sulfhydryl oxidases oxidize thiols in proteins during folding, reducing oxygen as the terminal electron acceptor (Fass). The human members of the sulfhydryl oxidase family that have been discovered to date exist as two functional isoforms produced through alternative splicing of ~100 amino acids before the 3'-UTR (Giorda et al.). The shorter protein generally resides in the cytoplasm while the longer protein contains a transmembrane domain and resides in the ER and/or mitochondria. Due to the difficulty in expressing and isolating the long form of each group member assumptions have been made but not validated for the individual functions of the two isoforms. Further studies need to be performed to understand the protein-substrate interactions that exist between these two isoforms, using peptide specific antibodies and/or thiol trapping experiments.

Disulfide bonds are required for the stability and function of many proteins. Comparative mutagenesis studies by the Fass and colleagues using Ero1p and Erv2p as sulfhydryl oxidase representatives have revealed common mechanistic themes. Each member of the family contains a CXX_nC motif (where X represents any amino acid and n represents varying distances between C) that directly interacts with the flavin adenine dinucleotide (FAD) cofactor, called the “active di-cysteine” to transfer electrons from the oxidation of sulfhydryl groups during protein refolding (Vitu et al.). Two additional CXXC motifs assist in directing protein-substrate interactions. One CXXC that is directly downstream of the “active di-cysteine” assists in the protein-substrate interaction (Vitu et al.). A second termed the “shuttle di-cysteine” is situated outside of the four-helix-bundle core on a flexible hinge that assists in directing proteins from the solvent to the active site (Vitu et al.). Until recently, the majority of sulfhydryl oxidase research has focused on the biochemical properties of these proteins to efficiently form disulfide bonds. Although interest in the role of sulfhydryl oxidases in diseases progression has recently intensified in part due to their expression in cancers and heart disease (Katchman, Antwi, Hostetter, Demeure, Watanabe, Decker, Miller, Hoff, and Lake; Morel et al.; Mebazaa et al.).

Quiescin Sulfhydryl Oxidase 1

QSOX1 belongs to the family of FAD-dependent sulfhydryl oxidases that are expressed in all eukaryotes sequenced to date (Thorpe et al.). 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 (Coppock, Cina-Poppe, and Gilleran; Coppock et al.; Heckler et al.). 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 (Coppock and Thorpe).

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 (Figure 1) (Thorpe et al.). 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 (Coppock, Cina-Poppe, and Gilleran; Vitu et al.; Alon, Heckler, et al.). QSOX1-L contains a predicted transmembrane domain that is not present in QSOX1-S due to alternative splicing (Figure 1) (Alon, Heckler, et al.). 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, a balance often altered in cancer cells (Hoober et al.). Since the initial discovery of QSOX1 the

majority of research to date has focused on detailing the sulfhydryl oxidase activity for disulfide bond formation in proteins. Thorpe and colleagues revealed the ability of QSOX1 to efficiently generate disulfide bonds into proteins during folding at rate of 1000 per minute with a K_M of 150uM per thiol (Heckler et al.; Alon, Grossman, et al.; Alon, Heckler, et al.). 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.

Redox state and redox regulation are important in embryo and fetal development as well as transcriptional and post-translational regulation in tumor progression (Andrade, Stolf, Debbas, Rosa, Kalil, Coelho, and Laurindo). In this context, QSOX1 may be involved in regulating the thiol-disulfide exchange reactions during development helping to maintain an adequate redox state. Indeed, Portes and colleagues have found in mice during development, QSOX1 expression is restricted mainly to mesoderm and ectoderm derived tissues and that QSOX1 expression seems to be developmentally regulated, increasing with tissue maturation. Further studies involving QSOX1 and its role in development could also allow us to infer its functional significance in tumor cells. As it is very common for developmentally regulated proteins to provide growth and metastatic advantages to tumor cells. Numerous receptor tyrosine kinases, epithelial-to-mesenchymal transition related transcription factors and basement membrane degrading proteases such as MMPs are activated in response

to H_2O_2 (Wu). The increase in QSOX1 expression in tumor cells could lead to the more oxidative environment commonly observed in tumor cells and surrounding stroma.

In contrast, work by Morel and colleagues proposed that expression of QSOX1 protects MCF7 breast tumor cells from oxidative stress-induced apoptosis (Morel et al.). They show that expression of QSOX1 directly slows the growth of MCF7 cells and that it correlates with an increase in cell survival after treatment with H_2O_2 (Morel et al.). Initially these findings suggested that increased QSOX1 expression in tumor cells may allow them to actively evade cellular apoptotic mechanisms mediated by reactive oxygen species. As research into the role of QSOX1 in tumor cells progressed the importance of this paper, as a central hypothesis possibly explaining why we see an overexpression of QSOX1 in tumor cells, failed to satisfy follow up testing. Loss of QSOX1 in MCF7 cells did not result in an increase in reactive oxygen species as measured by DCFDA staining nor did we see that the loss of QSOX1 resulted in an increase in apoptosis.

In direct support of our discover of QSOX1 in pancreatic tumor cells Song and colleagues, through microarray analysis, discovered that QSOX1 is expressed early in prostate cancer progression (Song et al.). They found that the loss of NKX3.1 expression correlates with an increase in QSOX1 expression in prostate tumors. NKX3.1 is a homeobox transcription factor and a known tumor suppressor that is exclusively

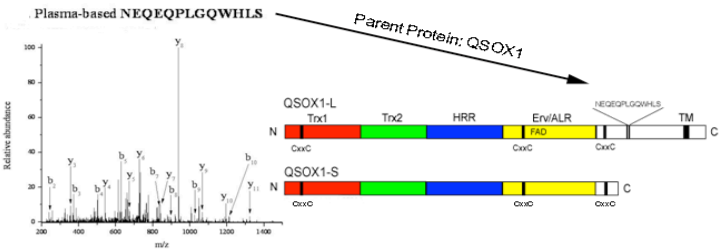
expressed in luminal epithelial cells of the prostate. QSOX1 has been shown to be highly expressed in early stages of prostatic neoplasia and throughout prostate cancer progression, but was not present in normal prostate (Song et al.; Ouyang). NKX3.1 expression is decreased or absent in early stages of prostate tumor development and absent in up to 80% of metastatic prostate tumors (Ouyang). Since loss of NKX3.1 results in over-expression of QSOX1, it further emphasized the need to uunderstand the role QSOX1 activity may play in early tumorigenesis (Song et al.).

While the expression of QSOX1 in tumor cells has been observed in numerous microarray studies the majority of these groups have not attempted to determine the function of QSOX1 in normal or tumor cells. Thus in this thesis I strove to analyze the functional advantage that QSOX1 provides tumor cells through the analysis of the hallmarks of cancer traits detailed in the introduction.

Plasma: Patient and Normal Samples

3 KDa Ultrafiltration: Remove overabundant proteins that mask potential biomarkers.

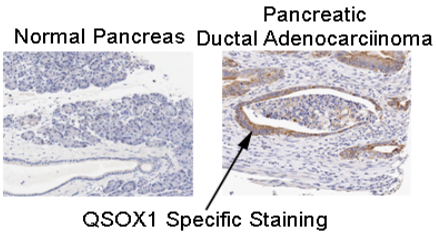
LC-MS/MS: Separate and identify proteins/peptides. Identify parent protein through NCBI or SWISSPROT.



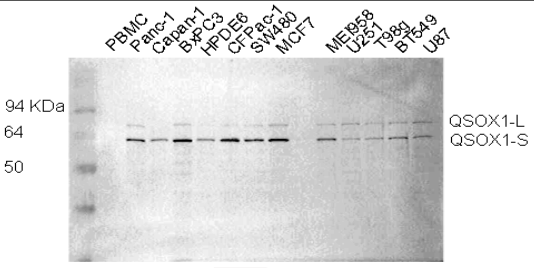
Antwi et al. 2009

Identify proteins/peptides that are expressed in patients but not in normal samples.

Patient Code	Diagnosis	Peptides
P3001	Ductal Adenocarcinoma	QSOX1
P3003	Ductal Adenocarcinoma	Not found
P3004	Ductal Adenocarcinoma	Not found
P1010	Ductal Adenocarcinoma	QSOX1
P1013	Ductal Adenocarcinoma	QSOX1
P1015	Ductal Adenocarcinoma	QSOX1
P1016	Ductal Adenocarcinoma	QSOX1
P1019	Ductal Adenocarcinoma	QSOX1
P1023	Ductal Adenocarcinoma	Not found
P1026	Ductal Adenocarcinoma	Not found
P1027	Ductal Adenocarcinoma	QSOX1
P1032	Ductal Adenocarcinoma	QSOX1
P1034	Ductal Adenocarcinoma	QSOX1
P1037	Ductal Adenocarcinoma	QSOX1
P1038	Ductal Adenocarcinoma	Not found
P3010	Ductal Adenocarcinoma	QSOX1
P1041	Ductal Adenocarcinoma	Not found
P1043	Ductal Adenocarcinoma	QSOX1
P4003	Ductal Adenocarcinoma	Not found
P4005	Ductal Adenocarcinoma	QSOX1
P4008	Ductal Adenocarcinoma	QSOX1
		14/21



QSOX1 is overexpressed in a variety of tumor cell lines but weakly expressed in a transformed normal cell lines.



What advantages does the expression of QSOX1 in tumor cells provide?

Knockdown QSOX1 using shRNA and evaluate cellular proliferation, cell cycle regulation, apoptotic resistance and invasive/ metastatic properties.

Figure 1. Our experimental outline of the discovery and confirmation of QSOX1 as a pancreas specific tumor cell marker. QSOX1 was initially discovered in 3 kDa filtered pancreatic tumor patient plasma. The subsequent filtered plasma, containing peptides and proteins 3 kDa and smaller, was then analyzed using LC-MS/MS to identify peptides that are unique to patient plasma but not found in normal plasma. A short peptide NEQEQPLGQWHLS was repeatedly discovered (14 of 24 patients) in patient plasma and was not found to be present in normal plasma. This short peptide was mapped back to a larger parent protein QSOX1-L. To confirm out mass spectrometry data we performed IHC and western blot analysis using a polyclonal anti-QSOX1 antibody of pancreatic ductal adenocarcinoma surgically resected tissue and numerous tumor cell lines. Our IHC data revealed that QSOX1 is a pancreatic tumor cell specific marker and that QSOX1 is expressed at variable levels in numerous different tumor cell culture line (pancreas, breast, colon, lung and brain). The question remained, what advantage does QSOX1 provide to tumor cells?

CHAPTER 2

METHODS TO ANALYZE THE ROLE OF QSOX1 EXPRESSION IN PANCREATIC AND BREAST ADENOCARCINOMA.

Abstract

Our initial results indicated that QSOX1 is expressed in human pancreatic, breast, lung, and prostate adenocarcinomas but is not found in adjacent normal tissues. Subsequently we were able to show that QSOX1 is also expressed at higher levels in pancreatic and breast adenocarcinoma cell lines compared to transformed normal cell lines (Figure 1). Fortunately expression of QSOX1 in *in vitro* cell lines mimicked that of our *in vivo* findings allowing us to use established cell lines to analyze the role of QSOX1 in tumor cells. Using RNA interference (RNAi) technology to degrade QSOX1 RNA and reduce QSOX1 protein expression we analyzed the role of QSOX1 as it relates to the hallmarks of cancer.

Overview

Loss-of-function studies using RNAi technology has revolutionized our ability to understand the role of specific genes and proteins (Carthew and Sontheimer). In the present study we utilized short hairpin RNA (shRNA) over conventional short interfering RNA (siRNA) to inhibit the expression of QSOX1. Utilization of shRNAs over siRNA provided us with three key features that gave us improved and reproducible results (a)

increased RNA degradation of QSOX1 (b) long term loss of QSOX1 (c) a DNA based system (shRNA) over an RNA based system (siRNA) alleviating concerns of RNase degradation providing highly reproducible results.

RNAi machinery can be programmed exogenously or endogenously through the utilization of double stranded RNA (Carthew and Sontheimer; Silva et al.). The most well characterized endogenous mechanism of gene inhibition is micro-RNA (miRNA) genes (Carthew and Sontheimer). In mammalian cells miRNAs are transcribed in the nucleus by RNA polymerase II leading to the generation of a long primary polyadenylated RNA (pri-miRNA) (Carthew and Sontheimer). The pri-miRNA is then recognized and processed by the RNase III enzyme Drosha. Drosha cleaves the hairpin to produce a pre-miRNA approximately 70-90 nucleotides in length with a 2-nucleotide 3' overhang (Carthew and Sontheimer). Proper processing of the pre-miRNA structure signals Exportin-5 to transport the pre-miRNA into the cytoplasm (Carthew and Sontheimer). In the cytoplasm the pre-miRNA structure is recognized and further processed by the enzyme Dicer to produce a mature miRNA of approximately 22 nucleotides. The mature miRNA is next recognized by the RNA induced silencing complex (RISC) that then targets and degrades your gene of interest leading to RNA and protein degradation (Carthew and Sontheimer; Silva et al.). Our current shRNA technology is designed

to function in the same manner as miRNA as well as utilizing key features found in strong miRNA such as miR-30 (Silva et al.).

To analyze the role of QSOX1 in pancreatic and breast adenocarcinoma we generated multiple shRNAs targeting QSOX1 in a pLK0.1 lentiviral vector. The establishment of stable QSOX1 knock-down cell lines in pancreatic and breast adenocarcinoma provided us with a robust and long term knock-down model which we were able to take advantage of in our efforts to study the role of QSOX1 as it relates to the hallmarks of cancer. In the current chapter we describe the methods used to analyze how the loss of QSOX1 affect pancreatic and breast adenocarcinoma cell lines.

Material and Methods

Cell culture

Pancreatic adenocarcinoma BxPC3, PANC-1, CFPac-1, MiaPaca-2 and Capan1 cancer 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). All cell lines were grown at 37^0C with 5% CO_2.

Breast adenocarcinoma MCF7, MDA-MB-468, MDA-MB-453, BT549 and MDA-MB-231 cancer cell lines were cultured in DMEM with 10% fetal bovine serum (FBS) (Gibco). Immortal human non-tumorigenic

breast epithelial cells (MCF10A) were cultured in Clontech KGM-2 karotinocyte media (Gibco). All cell lines were grown at 37^0C with 5% CO_2.

All cell lines tested negative for mycoplasma contamination using, Venor GeM Mycoplasma Detection Kit, (Sigma).

Immunohistochemistry (IHC) of Pancreatic Tissue

Tissue from patients who underwent surgical resection for PDA were formalin-fixed and paraffin-embedded. The tissue blocks were sectioned at 5 μm thickness, transferred to slides and dried overnight at room temperature. The slides were dewaxed, rehydrated and antigen retrieved on-line on the Bond autostainer (Leica Microsystems, Inc., Bannockburn, IL). The 5 μm cut slides were baked at 60 °C for 60 min and subjected to heat induced epitope retrieval, in a peroxidase free environment, using a proprietary citrate based retrieval solution for 20 min. The slides were incubated for 30 min with rabbit polyclonal anti-QSOX1 at 1:75 (Proteintech Group, Inc.,Chicago, IL). Each section was visualized using the Bond Polymer Refine Detection kit (Leica) using diaminobenzidine chromogen as substrate. Dr. Hostetter, a board-certified pathologist, evaluated each section using a standard scoring based on stain intensity (0-3) with 0 indicating no staining and 3 indicating strong staining. Stain localization in tumor cells were nuclear, cytoplasmic or membranous.

Immunohistochemistry (IHC) of Breast Tissue and Scoring of Staining Intensity

Breast tumor microarray slides were generated from 153 different breast cancer patients. Each patient's tumor was represented in triplicate on the slides. Immunohistochemistry on breast tumor tissue microarray (TMA) samples was performed exactly as previously described for pancreatic tissue. After staining the TMA slides with anti-QSOX1 rabbit polyclonal antibody, a board certified pathologist (Dr, Idris Tolgay Ocal) scored the staining pattern as i) the percentage of cells with IHC staining for QSOX1 protein expression in the core tumor tissue sample (0: no staining, 1: 1-33%, 2: 34-66%, 3: 67-100%) and ii) the intensity of the antibody stain (0: no staining, 1: weak, 2: moderate, 3: strong staining intensity).

Statistical Assessment of QSOX1 IHC with Molecular Subtypes of Breast Cancer

There were 153 patient tissue samples in triplicate stained with anti-QSOX1 rabbit polyclonal Ab (Proteintech). IHC staining was scored by a board certified pathologist (I.T.O.). The amount and intensity of QSOX1 staining/expression was scored on a scale of 0-3. The first IHC score number represents the percentage of cells staining (0: No staining, 1: 1-33%, 2: 34-66%, 3: 67-100%), and the second represents intensity (0:

No staining, 1: weak, 2: moderate, 3: strong staining intensity). We grouped the scores into 4 categories: 0, 11/12/21, 22/13/31 and 32/33/23.

To evaluate the relationship between markers and QSOX, Pearson's chi-square test was performed. Using 2-sided *P* values, statistical significance will be set at $P \leq 0.05$.

Generation of Short Hairpin (sh) RNA and Lentiviruses Production

Three different shRNA for QSOX1 were obtained through DNASU (http://dnasu.asu.edu) already in the lentiviral pLKO.1-puromycin selection vector. QSOX1 sh742, 5'-CCGG*GCCAATGTGGTGAGAAAGTTT*CTCGAGAAACTTTCTCACCACATTGGCTTTTTG - 3' (sense), QSOX1 sh528, 5'-*CCGGACAATGAAGAAGCCTTT* - 3' (sense), QSOX1 sh616, 5'-*TCTAGCCACAACAGGGTCAAT* -3' (sense) and shScramble with target sequence 5' –*TCCGTGGTGGACAGCCACATG* – 3' 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 as indicated in sh742.

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^oC, 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 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^{o}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 sh lentivirus were collected every 24 hours for a total of 72 hours, combined and stored at -20^{o}C.

Generation of shQSOX1-Transduced Tumor Cell Lines

Stable transduction of sh742, sh528, sh616 and shScramble into BxPC-3, Panc-1, MCF-7 and BT549 cell lines was performed by first seeding the cells at 8 x 10^5 cells/well in a 6 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 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, CFPac1, MCF10A, MCF7, MDA-MB-468, MDA-MB-453, BT549 and MDA-MB-231 cells as well as pancreatic cancer patients 1010 and 1016 tumor and adjacent normal enzymatic supernatant. Cell lysates were generated by harvesting 2.5 x 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 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 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. 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 x 10^3 cell/well in a 96-well plate in triplicate and 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.

Annexin V / Propidium Iodide Apoptosis Analysis

Apoptosis analysis was performed according to the manufacturers 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 1x10^6 cell/ml in 1x Annexin V Binding Buffer. Next, 1x10^5 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.

Cell Cycle Analysis

Cell cycle analysis was measured by flow cytometry (Becton Dickinson FACScalibur Flowcytometer) using propidium iodide (Invitrogen). BxPC3 and Panc-1 cells were seeded at equal densities in a $25cm^2$ flask until they reached 60-80% confluency. Cells were then removed from the flask using trypsin (Mediatech Inc.), pelleted and washed in cold 1xPBS twice. The cells were counted using a hemacytometer and normalized to $1x10^6$ cell/ml in 1xPBS. Cells were pelleted and resuspended in equal amounts of RNAse Digestion Buffer (2mg/ml of RNAse-A (Sigma) in 1.12% Na-Citrate) and Staining Buffer (100ug/mL Propidium Iodide in 0.2% Triton X-100 and 0.1% Na-Citrate). The samples were stored on ice and analyzed within one hour of collecting. Each sample was performed in triplicate.

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 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^{o}C for 3 min; PCR Cycling (40 cycles) 1.) 95^{o}C, 30 sec. 2.) 55^{o}C, 30 sec. 3.) 72^{o}C, 1 min; Melt Curve (Dissociation Stage). The primer sequences for the genes analyzed are: GAPDH Forward 5' – GGCCTCCAAGGAGTAAGACC; GAPDH Reverse 5' – AGGGGTCTACATGGCAACTG; QSOX1-S Forward 5' - TGGTCTAGCCACAACAGGGTCAAT; QSOX1-S Reverse 5' - TGTGGCAGGCAGAACAAAGTTCAC; QSOX1-L Forward 5' - TTGCTCCTT GTCTGGCCTAGAAGT ; QSOX1-L Reverse 5' - TGTGTCAAAGGAGCTCTCTCTGTCCT
; MMP-2 Forward 5' – TTGACGGTAAGGACGGACTC; MMP-2 Reverse 5' – ACTTGCAGTACTCCCCATCG; MMP-9 Forward 5' – TTGACAGCGACAAGAAGTGG; MMP-9 Reverse 5' –

CCCTCAGTGAAGCGGTACAT. Each reaction was performed in triplicate with the data representing the averages of one experiment.

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 two tailed T-test.

Matrigel and non-Matrigel Coated Invasion Assay

Invasion assays were performed using BD BioCoat™ BD Matrigel™ as well as non-Matrigel™ control 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 chamber in serum free DMEM. The outer chamber contained 10% FBS in DMEM. BxPC3, Panc-1, MCF-7 and BT549 cells were incubated for 24 hours (48 hours for BT549 cells) at 37^0C, 5% CO_2. Cells that invaded through the Matrigel and migrated through the pores onto the bottom of the insert were fixed in 100% methanol and then stained in hematoxylin (Invitrogen). The total number of invading cells were determined by counting the cells on the underside of the insert from 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 determined using the Student's two tailed T-test. Results presented are from one of three independent experiments.

Matrigel Invasion Recovery Assay

Invasion assays were performed using BD BioCoat™ BD Matrigel™ and non-Matrigel™ control 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 x 10^4 cells/well were seeded into the inner matrigel chamber in serum free DMEM. The outer chamber contained 10% FBS in DMEM. MCF7, BT549, and MDA-MB-231 cells were incubated for 72, 48 and 24 hours, respectively at 37^0C, 5% CO_2. For invasion rescue assays MCF7 and BT549 cells were incubated with 50nM rQSOX1 as well as catalytically inactive mutant rQSOX1 (rQSOX1-AA). Cells that invaded through the Matrigel and migrated through the pores onto the bottom of the insert were fixed in 100% methanol and then stained in hematoxylin (Invitrogen). The total number of invading cells were determined by counting the cells on the underside of the insert from triplicate wells (6 fields per insert) at 20x and 40x magnification. The extent of invasion was expressed as the mean +/- S.D. Significance was determined using the Student's two tailed T-test. Results presented are from one of three independent experiments.

Gelatin Zymography

The identification of matrix metalloproteinases (MMP) was performed using gelatin zymography. Zymography experiments were performed as follows. Untreated BxPC3, Panc-1, MCF-7 and BT549 cells as well as shRNA 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 collecting the serum free DMEM, protein concentration was 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) (Snoek-van Beurden and den Hoff). 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 (Table 1). 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.

Conclusion

Understanding the role of QSOX1 in normal or tumor cells prior to this study was very minimal (Andrade, Stolf, Debbas, Rosa, Kalil, Coelho, and Laurindo; Morel et al.; Song et al.). The goal of our initial studies was to determine if QSOX1 provided any advantages to tumor cells. To determine if the expression of QSOX1 could be directly linked to the hallmark traits of tumors we used loss-of-function studies. Due to the lack of background information on the role of QSOX1 we chose establish an *in vitro* model and use broad diagnostic measures to determine if the expression of QSOX1 enabled growth, apoptosis, cell cycle regulation and invasion.

CHAPTER 3

QUIESCIN SULFHYDRYL OXIDASE 1 (QSOX1) PROMOTES INVASION OF PANCREATIC TUMOR CELLS MEDIATED BY MATRIX METALLOPROTEINASES.

Abstract

We previously mapped a peptide in plasma from pancreatic ductal adenocarcinoma (PDA) patients back to an over-expressed QSOX1 parent protein. In addition to over-expression in pancreatic cancer cell lines, 29 of 37 patients diagnosed with pancreatic ductal adenocarcinoma expressed QSOX1 protein in tumor cells, but QSOX1 was not detected in normal adjacent tissues or in a transformed, but non-tumorigenic cell line. The expression of QSOX1 in tumor cells but not in normal cells suggests that QSOX1 provides a functional advantage to tumor cells that is not needed in normal cells. Chapter 3 begins to assess the role of QSOX1 in tumors by suppressing QSOX1 protein expression using short hairpin RNA (shRNA) in 2 pancreatic cancer cell lines, BxPC-3 and Panc-1. Tumor cell growth, cell cycle, apoptosis, invasion and MMP activity were evaluated. QSOX1 shRNA suppressed both short and long isoforms of the protein showing a significant effect on cell growth, and to a lesser extent on cell cycle and apoptosis. However, QSOX1 shRNA dramatically inhibited the abilities of BxPC-3 and Panc-1 pancreatic tumor cells to invade through Matrigel in a modified Boyden chamber assay. Mechanistically, gelatin zymography indicated that QSOX1 plays an

important role in activation of MMP-2 and -9. Taken together, our results suggest that the advantage that QSOX1 provides to tumors is post-translational activation of MMP-2 and -9.

Overview

Pancreatic ductal adenocarcinoma (PDA) is a disease that carries a poor prognosis primarily due to the advanced stage of the disease upon diagnosis. There are over 33,000 patients diagnosed with PDA in the United States annually and over 80% of those patients present with advanced, stage III tumors, and are usually not candidates for surgery at the time of diagnosis (Koorstra et al.). However, even if pancreatic cancer is surgically resected in stage I or II, it may recur at a metastatic site (Bardeesy and DePinho; Almhanna and Philip). Currently, patients diagnosed with pancreatic ductal adenocarcinoma have less than a 5% chance of surviving past five years due to the late onset of symptoms, the highly aggressive nature of PDA and the lack of non-invasive diagnostic markers that would allow for early detection (Wong and Lemoine; Koorstra et al.).

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) (Figure 1) (Antwi, Hostetter, Demeure, Katchman, Decker, Ruiz, Sielaff, Koep, and Lake). Subsequently, we found that QSOX1 is over-expressed in tumor tissue from pancreatic cancer patients, but not adjacent normal tissue (Figure 2B

& 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.

In the present study, we have begun to analyze the biology of QSOX1 in tumors using pancreatic tumor cell lines BxPC3 and Panc-1. We knocked down QSOX1-S and –L protein expression using shRNA to determine if suppression of QSOX1 affected cell growth, cell cycle, apoptosis, invasion and MMP activity. QSOX1 knock-downs slowed tumor cell proliferation and affected cell cycle and apoptosis. We also observed a dramatic decrease in tumor cell invasion *in vitro* 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 role for QSOX1 in invasion and metastasis.

RESULTS

Detection of QSOX1 by Immunohistochemistry and Western blot

To begin to determine the frequency of expression of QSOX1 in human PDA, QSOX1 expression was assessed in 4 different pancreatic tumor cell lines, an immortal non-tumorigenic cell line, HPDE6, 37 tumor tissue sections from patients with PDA, and fresh frozen tumor tissue and adjacent normal tissue from two patients, 1016 and 1010 (Figure 2B, C, and D). 29 of 37 tumor tissues were positive for QSOX1 expression by

immunohistochemistry (IHC), 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 (Figure 2C). Our results revealed that QSOX1-S is the dominant splice variant expressed in tumor tissue. One of the adjacent normal tissue samples (1016) showed a high level of QSOX1 expression, but it is possible that there was tumor present in the adjacent tissue, 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-tumorigenic pancreas epithelial cell line, shows weak expression of QSOX1-S and no detectable expression of QSOX1-L (Figure 2D).

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 (Figure 2B & C). Second, QSOX1 protein expression in adjacent normal 1010, 1016, and HPDE6, a non-tumorigenic pancreatic duct cell line, is weaker than 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.

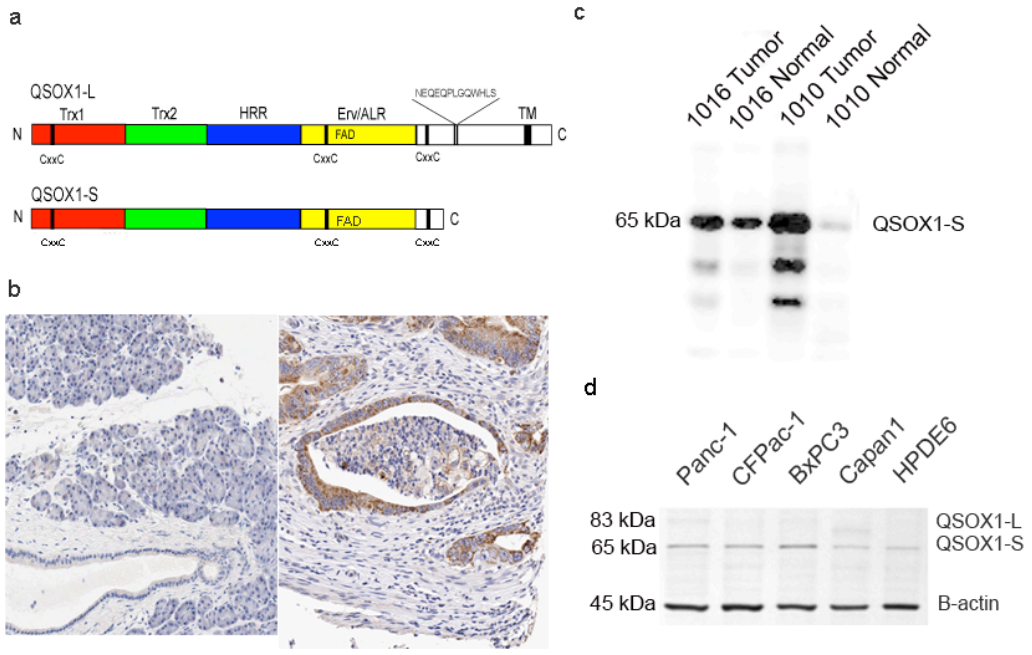


Figure 2: QSOX1 is highly expressed in tumor cell lines but is not expressed in adjacent normal cells. Previously, our lab discovered a short peptide, NEQEQPLGQWHLS, in patient plasma through LC-MS/MS. We were able to map this short, secreted peptide back to an understudied parent protein, QSOX1-L. A.) 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, maps back to QSOX1-L, and found to be secreted in pancreatic cancer patients but not in normal samples. The commercially available antibody recognizes the first 329 amino acids of both QSOX1-S and -L. B.) Immunohistochemistry of normal (left) and tumor (right) pancreatic tissue sections that have been stained with the anti-QSOX1 showing tumor specific staining in pancreatic ducts but not in adjacent non-tumor cells. C.) Western blot analysis of patient tumor as well as adjacent normal tissue indicates that QSOX1-S is the dominant splice variant expressed. D.) Western blot showing QSOX1 expression in transformed normal pancreatic cells (HPDE6) and Human Pancreatic 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.

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 constructs are active in both cell lines, figure 3A and 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 demonstrated 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 (Figure 3A & B).

ShQSOX1-transduced BxPC3 and Panc-1 cells exhibited a decrease in cell growth compared to shScrambled controls in an MTT assay (Figure 3C). 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

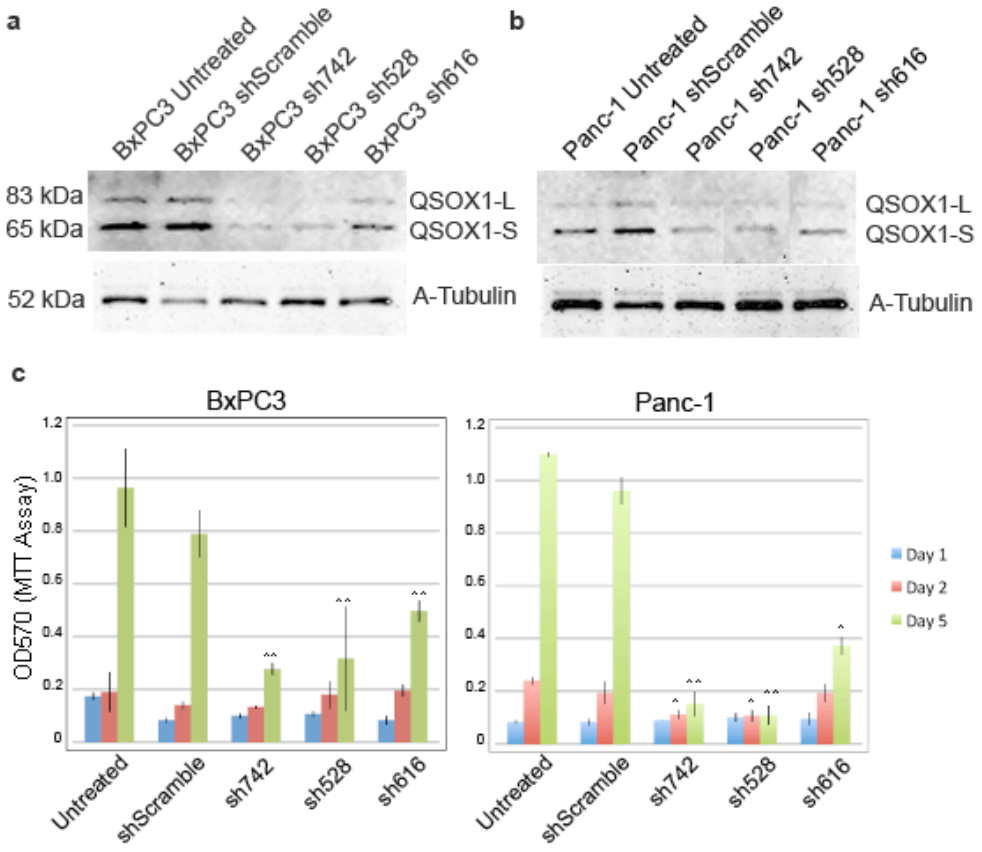


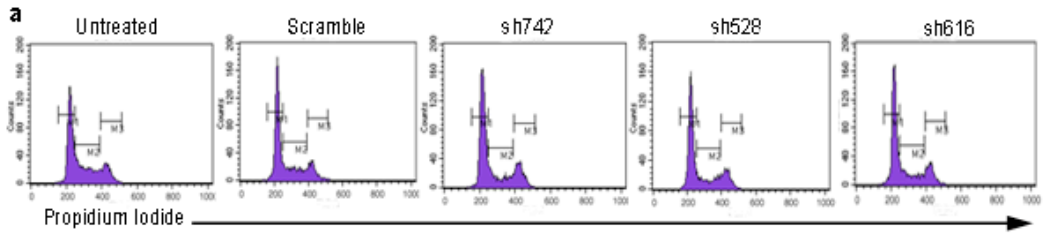
Figure 3: Reduced expression of QSOX1 in BxPC3 and Panc-1 cells leads to a significant decrease in cell growth. To determine the phenotype presented due to the expression of QSOX1 in tumor cells we employed shRNA specific to QSOX1 to reduce the expression of QSOX1 in A.) BxPC3 (Percent Decrease in sh742 – 56%; sh528 – 40%; sh616 – 28%) and B.) Panc-1 (Percent Decrease in sh742 – 64%; sh528 – 46%; sh616 – 18%) cells and further evaluated cell growth, cell cycle, apoptosis, and invasion/metastasis. Western blots have been cropped and full images can be viewed in S3. 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.

days 1 and 2 there was no change, 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 an 84%, 88% and 61% decrease in cell growth as measured by mitochondrial respiration.

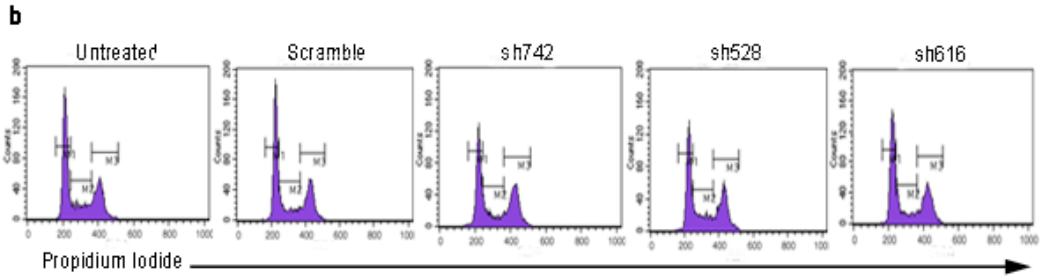
Cell Cycle and Apoptosis Analysis

Previous work has correlated QSOX1 expression with the quiescent stage, G_o, of the cell cycle (Thorpe et al.). This led us to hypothesize that shQSOX1-mediated decrease in cell proliferation was the result of abnormal regulation of the cell cycle or an increase in apoptosis. To address this hypothesis, propidium iodide (PI) was used in flow cytometry to evaluate the effects of shQSOX1 on cell cycle. Our results indicate that suppression of QSOX1 expression marginally modulated cell cycle in both BxPC3 and Panc-1 compared to our untreated and scrambled control (Figure 4A & B). The results show that the reduced expression of QSOX1 on cell cycle could be cell line dependent. BxPC3 showed an increase in G_1 and a significant decrease in S, while Panc-1 cells showed a significant decrease in G_1 but no changes in S (Figure 4A & B).

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 (Figure 5).



Percent BxPC3 Cells in G_1, S, and G_2/M					
	Untreated	shScramble	sh742	sh528	sh616
M1 (G_1)	50.44	60.79	62.76	61.33	62.41
M2 (S)	32.63	25.19	18.74	20.71	21.27
M3 (G_2/M)	16.28	13.17	17.82	17.1	15.74



Percent Panc-1 Cells in G_1, S, and G_2/M					
	Untreated	shScramble	sh742	sh528	sh616
M1 (G_1)	52.08	50.38	43.86	41.73	48.31
M2 (S)	19.2	17.61	18.03	22.43	19.06
M3 (G_2/M)	29.16	32.55	38.66	27.73	33.22

Figure 4: Reduced QSOX1 expression leads to alterations in the cell cycle. Cell cycle analysis, using propidium iodide, was performed on A.) BxPC3 and B.) Panc-1 cells in which QSOX1 protein expression was reduced using shRNA. Plots show representative data sets for gated samples of Untreated, Scramble, sh742, sh528 and sh616 from one experiment performed in triplicate. The percentages represent the number of cells in each stage of the cell cycle G1 (M1), S (M2), and G2/M (M3) phase. Data was calculated using Cell Quest Pro. This experiment was repeated in triplicate.

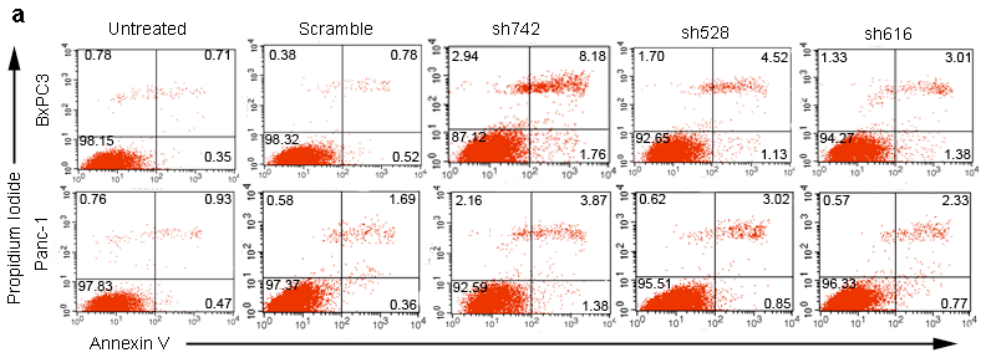


Figure 5: Reduced expression of QSOX1 in BxPC3 and Panc-1 cells leads to an increase in annexin V/ propidium iodide positive cells. A.) Apoptosis Analysis (Annexin V/Propidium Iodide) was performed on BxPC3 and Panc-1 cells in which QSOX1 was reduced using shRNA. Plots show representative data from one 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 (Lower Right), annexin V/propidium iodide double positive (Upper Left), or propidium iodide positive (Upper Right). Data was calculated using Cell Quest Pro software.

Compared to untreated and shScramble a consistent increase 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 not entirely account for the dramatic decrease in cellular proliferation. This data also agrees with viable cell counts revealing a largely insignificant decrease in shQSOX1 viable cells compared to untreated and shScramble controls.

Role of QSOX1 in Tumor Cell Invasion and Motility

For a tumor cell to invade other tissues as part of the metastatic process, the cell must first degrade basement membrane components such as laminin, collagen and fibronectin before it can migrate into the blood stream and re-establish itself in a distant organ (Bacac and Stamenkovic). 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, 8μm pore inserts. Inserts were placed into wells containing 10% FBS in DMEM. After 18 hours of incubation, tumor cells that had degraded Matrigel and migrated through 8μm pores onto the underside of the insert were counted (Figure 6A & 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 nutrient rich media.

To determine if the decrease in the invasive capabilities of BxPC3 and Panc-1 cells treated with sh742 and sh528 was due to an inability to degrade the matrigel allowing for the tumor cells to invade or if the loss of QSOX1 leads to a decrease in cellular motility we performed an invasion assay using non-Matrigel coated PET membranes containing 8μm pore inserts. The untreated, scramble, sh742 and sh528 transduced cells were seeded at equal densities in serum free media in the upper chamber. The inserts were then placed in wells containing 10% FBS in DMEM. After 24 hours of incubation, tumor cells that had migrated through 8μm pores onto the underside of the insert were counted (Figure 7A & B). Our results demonstrate that the loss of QSOX1 significantly affects the motility of BxPC3 and Panc-1 cells. While, the decrease in tumor cell motility (non-Matrigel) strongly suggests that QSOX1 plays a role in cellular motility the results in figure 7 do not demonstrate the same dramatic decrease as shown with Matrigel (Figure 6) coated inserts suggesting that there are further mechanisms that are preventing the tumor cells from degrading the Matrigel and migrating to the nutrient rich media.

Mechanism of Invasion

Since knock-down of QSOX1 protein expression in pancreatic tumor cell lines decreases invasion through Matrigel, it was important to

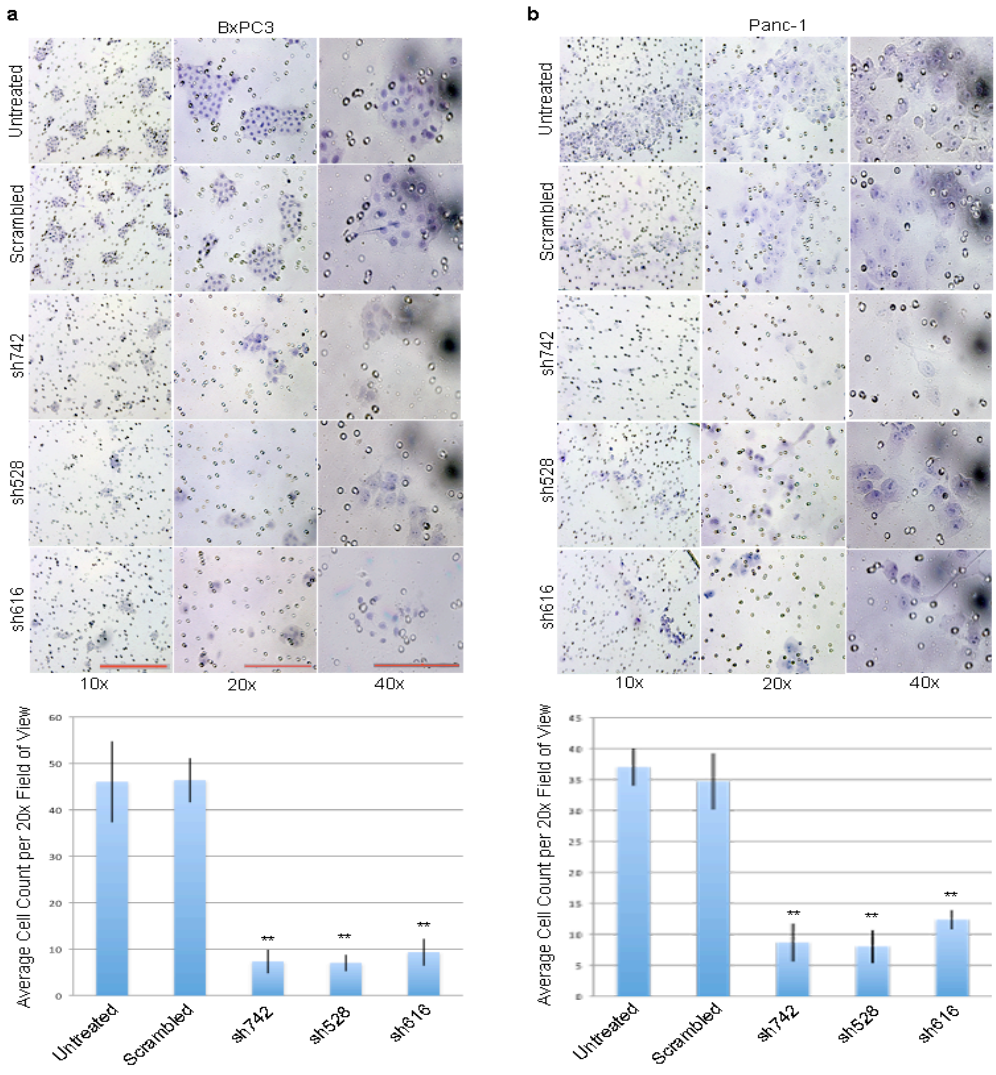


Figure 6: Reduced expression of QSOX1 in BxPC3 and Panc-1 cells leads to a significant decrease in in the ability to degrade Matrigel and invade into a nutrient rich media. A.) Untreated BxPC3 and B.) Untreated Panc-1 cells were treated with Scramble, sh742, sh528 and sh616 shRNA's specific for QSOX1 and seeded in the top chamber of Matrigel 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 and invade to the underside of the membrane, repectfully. 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 and invade through the membrane. Graphs represent average ± standard deviation (BxPC3 n = 6; PANC-1 n = 3), significance *, $P < 0.05$, **, $P < 0.005$.

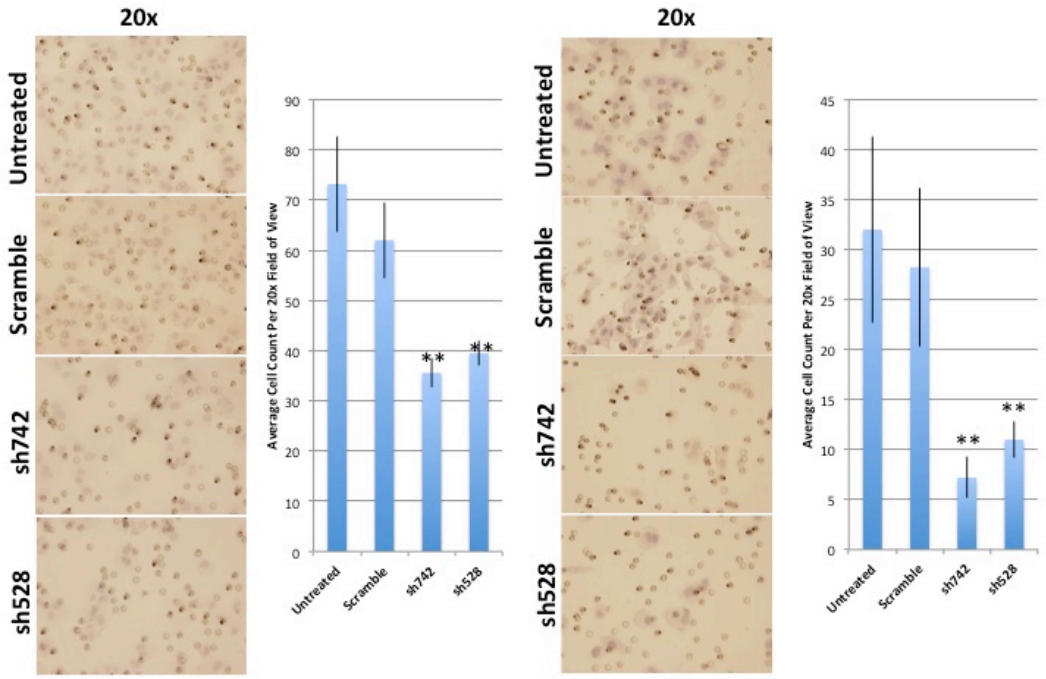


Figure 7: Reduced expression of QSOX1 in BxPC3 and Panc-1 cells leads to a significant decrease in cellular invasion. A.) Untreated BxPC3 and B.) Untreated Panc-1 cells were treated with Scramble, sh742 and sh528 shRNA's specific for QSOX1 and seeded in the top chamber of a non-coated invasion assay well and allowed to incubate for 18 hours. Representative 20x images are presented. In the BxPC3 sh742 and sh528 treated cells there was an 44% and 38% decrease in cells that were able to invade to the underside of the membrane, repectfully. While in Panc-1 sh742 and sh528 cells there was a 75% and 62% decrease in cells that were able to invade through the membrane. Graphs represent average ± standard deviation (BxPC3 n = 3; Panc-1 n = 3), significance *, $P < 0.05$, **, $P < 0.005$.

determine the mechanism of inhibition of the invasive process. MMP-2 and -9 are key contributors of invasion and metastasis in pancreatic cancer (Bardeesy and DePinho). Both pro-MMP-2 and -9 mRNA and protein levels are elevated in pancreatic tumors, and activated MMP-2 (a-MMP2) appears to be a key contributor of metastasis in PDA (Bardeesy and DePinho; Kessenbrock, Plaks, and Werb). Because QSOX1 has been suggested to be secreted into the extracellular matrix where MMPs are thought to be activated, we hypothesized that QSOX1 might help activate MMP-2 and -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 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 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).

Supernatants from BxPC3 cells transduced with sh742, sh528 and sh616 showed a 65%, 47% and 10% decrease, respectively, in pro-MMP9 compared to shScramble (Figure 8A). Supernatants from Panc-1 cells

tranduced with sh742, sh528 and sh616 showed a 70%, 56% and 15% decrease, respectively, in pro-MMP-2 (Figure 8B). Sh616 did not knock down QSOX1 as effectively as sh528 and sh742, and this is reflected in the cell growth, invasion and MMP activity. Thus, decreases in the proteolytic activity of MMP-2 and -9, using gelatin as a substrate, provide a mechanism for the QSOX1-mediated invasion through Matrigel.

To confirm our gelatin zymography results we used western blot analysis of BxPC3 and Panc-1 serum free conditioned media to probe for MMP-2 and -9 (Figure 8C). While our results indicate a slight decrease in MMP-2 and -9 (between 1-10% decrease using densitometry analysis) in BxPC3 and Panc-1 shQSOX1 treated cells it does not reflect the level shown using gelatin zymography. This could be explained as a difference between a functional assay, gelatin zymography, and a purely quantitative assay such as western blot.

To extend our hypothesis that QSOX1 is influencing MMPs 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 8D 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 activate MMP production, rather it post-translationally activates MMP activity. It also diminishes the possibility that shQSOX1 RNAs are suppressing MMP transcription due to off-target effects.

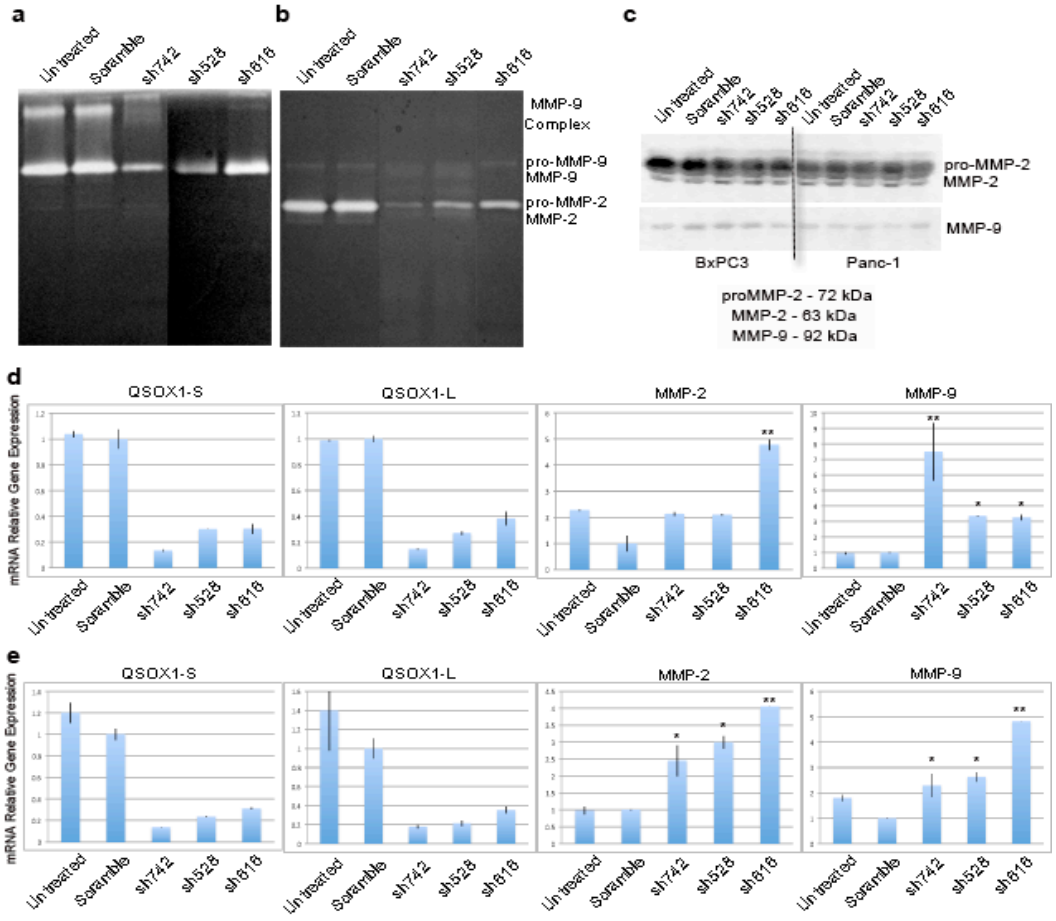


Figure 8: Reduced expression of QSOX1 leads to a decrease in secreted proMMP-9 in BxPC3 and proMMP-2 in Panc-1 cells. Gelatin zymography of A.) BxPC3 and B.) Panc-1 conditioned media showing a decrease in MMP-9 homodimers (MMP-9 Complex) (240 and 130 kDa), pro-MMP9 (92 kDa), pro-MMP2 (72 kDa) and active MMP-2 (a-MMP2, 66 kDa). Using Image J we were able to quantitify 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. Full images can be seen in S3. D.) The effect of shRNA mediated knockdown of QSOX1 on the expression of QSOX1-S, QSOX1-L, MMP-2, and MMP-9 in BxPC3 and 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.

Conclusion

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 (Koorstra et al.). Because most patients present with advanced metastatic disease, it is critical to understand the properties of invasive pancreatic tumors. The results presented suggest that QSOX1 is a commonly expressed protein in PDA making it a potential therapeutic target. To extend these findings we began to investigate why pancreatic tumors express QSOX1, and mechanistically, what advantage it affords tumors.

QSOX1 was previously reported by our group to be over-expressed in patients diagnosed with pancreatic cancer (Antwi, Hostetter, Demeure, Katchman, Decker, Ruiz, Sielaff, Koep, and Lake), and that a peptide from the QSOX1 parent protein is present in plasma from patients with PDA (Figure 1). In the present study we demonstrated for the first time that expression of QSOX1 in pancreatic tumor cells directly contributes to growth (Figure 3C) and an invasive and potentially metastatic phenotype (Figure 6 and 7) through the activation of MMP-2 and -9 (Figure 8A & B) through an as yet undetermined molecular mechanism.

The utilities of QSOX1 as a diagnostic and therapeutic target of pancreatic cancer remain to be determined. Preliminary IHC of breast, lung, prostate and colon cancer suggest that QSOX1 could be a pan tumor marker. If the function of QSOX1 is conserved despite the

histological origin of tumor, QSOX1 may be a broad therapeutic target. Our results underscore the need to further understand the role that QSOX1 plays in tumor and normal cells. Understanding the function of QSOX1 in multiple tumor types as well as the substrates in which QSOX1 interacts with could lead to effective treatment of advanced cancers.

CHAPTER 4

EXPRESSION OF QUIESCIN SULFHYDRYL OXIDASE 1 IS ASSOCIATED WITH A HIGHLY INVASIVE PHENOTYPE AND CORRELATES WITH A POOR PROGNOSIS IN LUMINAL B BREAST CANCER.

Abstract

Quiescin sulfhydryl oxidase 1 (QSOX1) oxidizes sulfhydryl groups to form disulfide bonds in proteins. Informatic analysis using the “Gene Expression Based Outcome for Breast Cancer Online” (GOBO) tool indicated high levels of QSOX1 RNA expression in Estrogen Receptor positive (ER+) subtypes of breast cancer. We confirmed this finding by evaluation of QSOX1 protein expression in breast tumors and in a panel of breast cancer cell lines. In addition, Kaplan Meyer analyses revealed QSOX1 as a highly significant predictive marker for both relapse-free and poor overall survival in Luminal B tumors, but not in other intrinsic subtypes. To investigate malignant cell mechanisms in which QSOX1 might play a key role, we suppressed QSOX1 protein expression using short hairpin (sh) RNA in ER+ MCF7 and ER- BT549 breast cancer cell lines. Suppression of QSOX1 protein dramatically slowed cell proliferation but did not significantly affect apoptosis or cell cycle regulation. Inhibition of QSOX1 did, however, dramatically inhibit MCF7 and BT549 breast tumor cells from invading through Matrigel in a modified Boyden chamber assay. Inhibition of invasion could be rescued by the exogenous addition

of recombinant QSOX1. Gelatin zymography indicated that QSOX1 plays an important role in activation of MMP-9 a key mediator of breast cancer invasive behavior. Taken together, our results suggest that QSOX1 is a novel biomarker for risk of relapse and poor survival in Luminal B breast cancer, and has a pro-invasive role in malignant progression through post-translational activation of MMP-9.

Overview

Breast adenocarcinoma is the most common cancer diagnosed in women throughout the world (Sgroi). In 2012, an estimated 226,870 new cases of invasive breast cancer are expected to occur among US women, and an estimated 39,510 breast cancer deaths (Society; Siegel, Naishadham, and Jemal). Despite significant advances in subtype classification of breast cancers, context-specific drivers of invasion and metastasis are still poorly understood. Improvements in screening and breast cancer awareness have increased the rate of early diagnosis yet the most successful prevention for women with a family history of breast cancer still remains to be preventative mastectomy (Talmadge and Fidler). The majority of breast tumors have a very slow growth rate, with an average doubling time of ±44 days (Talmadge and Fidler). Extrapolating this information lets us calculate that on average it takes 12 years for a primary tumor to reach 1cm (10^9 cells) in size, which currently is the lowest detectable limit (Talmadge and Fidler). During that time the tumor

often develops angiogenic and metastatic capabilities that contribute to a poor overall outcome.

Initially we reported the identification of a short peptide that maps back to the C-terminus of QSOX1 in plasma from pancreatic cancer patients (Antwi, Hostetter, Demeure, Katchman, Decker, Ruiz, Sielaff, Koep, and Lake). Subsequently, we found that QSOX1 is over-expressed in tumor tissue from pancreatic cancer patients, but not adjacent normal tissue (Katchman, Antwi, Hostetter, Demeure, Watanabe, Decker, Miller, Hoff, and Lake). To determine if QSOX1 is overexpressed in other tumor types we performed IHC on breast tissue microarrays and discovered that the expression of QSOX1 is specific to dysplastic cells as well. These findings led us to hypothesize that over-expression of QSOX1 might be functionally conserved between pancreatic ductal adenocarcinoma and breast adenocarcinoma, prompting further exploration of the function of QSOX1 as it relates to cancer (Hanahan and Weinberg).

In the current chapter we evaluated QSOX1 protein expression in breast adenocarcinoma cell lines MCF7 and BT549 and in a breast tumor tissue microarray. Using shRNA specific for QSOX1-S and –L, we assessed the effects of QSOX1 knockdown on cell growth, cell cycle, apoptosis, invasion and matrix metalloproteinase activity. The loss of QSOX1 significantly affected tumor cell proliferation and dramatically suppressed tumor cell invasion through Matrigel while the addition of exogenous catalytically active recombinant human QSOX1 (rhQSOX1)

rescued the invasive properties of both MCF7 and BT549 transduced with a lentivirus encoding shQSOX1. Further investigation into the mechanism of invasion revealed that QSOX1 is at least partially responsible for MMP-9 activity.

RESULTS

Expression of QSOX1 correlates with poor prognosis in patients with Luminal B breast cancer.

Bioinformatic analysis of QSOX1 transcript expression was assessed using data from the Gene expression based Outcome for Breast cancer Online algorithm (GOBO) (Ringnér, Fredlund, Häkkinen, Borg, and Staaf). GOBO analysis utilizes Affymetrics gene expression data curated from 1881 breast cancer patients with associated stage, grade, nodal status and intrinsic molecular classification based on the paradigm first reported by the Perou laboratory (Sørlie et al.). Expression of QSOX1 was significantly higher in ER+ tumors compared to ER- (p-value <0.00001), with the highest expression observed in Luminal A, Luminal B and Normal like subtypes, and lowest expression in HER2-enriched and basal-like tumors (Figure 9A & B). We performed a series of Kaplan Meier analyses to determine whether QSOX1 expression is associated with relapse free survival (RFS) and overall survival (OS) (Figure 9C & D). The results of these statistically significant analyses are summarized in Table 1. While elevated QSOX1 expression is not associated with survival when

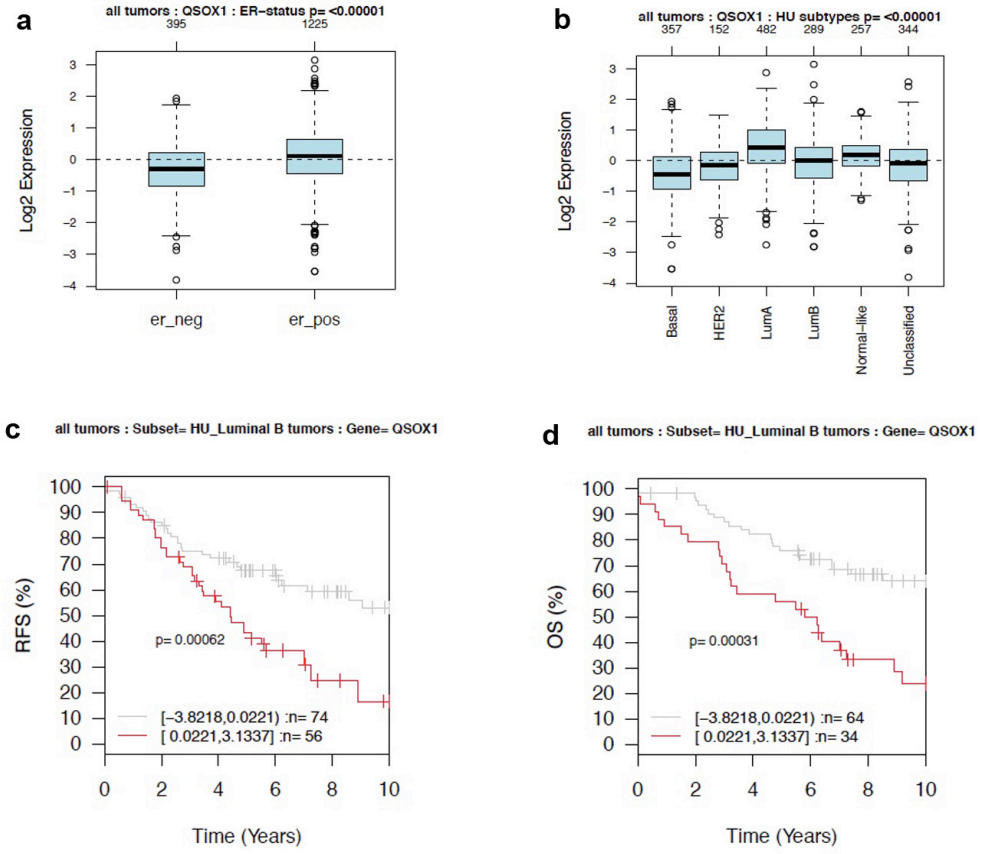


Figure 9. GOBO analyses of QSOX1 transcript expression among subtypes of breast cancer from over 1800 cases. **a)** Box plot analysis of QSOX1 mRNA expression in all tumors ER+ (n=1225) and ER- tumors (n=395) (p= <0.00001); **b)** Box plot analyses of QSOX1 expression among HU subtypes, Basal (n=357), HER2 (n=152), Luminal A (n=482), Luminal B (n=289), Normal-like (n=257) and untreated (n=344), (p= <0.00001). **c)** Kaplan Meier analysis of relapse free survival (RFS) in patients with Luminal B breast cancer expressing high (red line) and low (grey line) QSOX1 mRNA; High (n=56), low (n=74), (p=0.00062) and **d)** Overall survival (OS); High (n=34), low (n=64), (p=0.00031). Data obtained using GOBO: Gene expression based outcome for breast cancer online (http://co.bmc.lu.se/gobo).

	RFS (p-value)	Low (n)	High (n)	OS (p-value)	Low (n)	High (n)
All tumors	0.86755	477	437	***0.09682**	424	313
ER+	0.35126	354	384	***0.02481**	287	283
ER-	0.76167	120	46	0.46447	137	36
Luminal A	0.77820	84	177	***0.02507**	62	127
Luminal B	***0.00062**	74	56	***0.00031**	64	34
HER2	0.77143	44	27	0.73943	39	21
Normal Like	0.10710	68	111	0.45693	50	77
Basal-like	0.82086	113	30	0.50018	123	23
Grade1	0.16030	60	130	0.19165	51	88
Grade 2	0.50226	207	204	***0.04242**	163	152
Grade 3	0.30450	178	79	0.07095	197	65
Untreated	0.45345	261	156	***0.00109**	216	91

Table 1 Summary of the statistical analysis from GOBO: Gene Expression Based Outcome for Breast Cancer Online of mRNA expression for QSOX1 in breast cancer subtypes using relapse free survival (RFS) and overall survival (OS) as an endpoint. Each subtype was separated into two groups based on the mRNA expression levels of QSOX1. High and low refers to patients with low QSOX1 expression of mRNA ranging from (-3.8218, 0.0221) and patients with high levels of QSOX1 mRNA ranging from (0.0221, 3.1337) while n refers to the total number of patients sampled within the subtype indicated. A two-tailed ttest was performed to determine the statistical significance of QSOX1 expression within each individual subtype, expressed as the p-value.

considering all breast tumor subtypes together (Table 1), it is highly statistically associated with poor RFS (p=0.00062) and OS (p=0.00031) in Luminal B tumors (Figure 9C & D; Table 1). Elevated QSOX1 was also associated with reduced OS in luminal A tumors and is a poor predictor of OS for patients who did not receive systemic treatment (Table 1).

Evaluation of QSOX1 expression by Immunohistochemistry

Results from the GOBO transcript expression analysis fueled investigation of QSOX1 at the protein level in breast tumors. A breast tumor tissue microarray composed of breast tumors from over 150 different patients was stained with a rabbit anti-QSOX1 polyclonal antibody and scored by a board certified pathologist (I.T.O.). Figures 10A and B represent the pattern of QSOX1 expression observed in the TMA in an invasive ductal carcinoma (Figure 10A) and an invasive lobular carcinoma (Figure 10B). Statistical evaluation of QSOX1 expression by immunohistochemistry (IHC) demonstrated a strong association of ER+ tumors with a higher QSOX1 IHC score (Table 2), with no statistical association observed for QSOX1 expression and HER2+ tumors or cytokeratin markers of the basal-like subtype. This data is consistent with the correlation observed in the GOBO data. Interestingly, higher-grade tumors were associated with a higher QSOX1 IHC score (Table 2). Conversely, lower QSOX1 protein expression was significantly associated

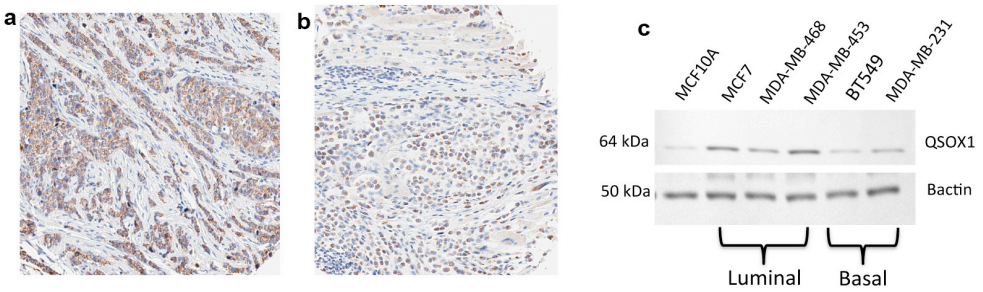


Figure 10. Protein expression of QSOX1 is specific for breast tumor cells in tissue (a, b) Immunohistochemistry of breast tumor tissue microarray samples showing positive staining for QSOX1 in tumor samples: **a)** invasive ductal carcinoma, ER+, PR+; **b)** invasive lobular carcinoma, ER+, PR-. Polyclonal antibody recognizes residues 1-329 of both QSOX1-S and –L. **c)** Western blot showing weak expression of QSOX1 in transformed, but non-tumor-forming MCF10A and human breast ductal carcinoma cell lines MCF7, MDA-MB-468, MDA-MB-453, BT549 and MDA-MB-231. β-actin loading control is shown below each lane.

	IHC score				p-value
	0 (n=17) %	11/12/21 (n=47) %	13/22/31 (n=24) %	23/33/32 (n=65) %	
Grade					*0.0003
1	53.3	42.2	25.0	10.8	
2	33.3	33.3	41.7	32.3	
3	13.3	24.4	33.3	56.9	
ER					*0.0013
+	80.0	89.1	73.9	55.4	
-	20.0	10.9	26.1	44.6	
HER2					0.0811
Positive/equivocal	11.8	6.4	29.2	14.1	
Negative	88.2	93.6	70.8	85.9	
CK5/6					0.0733
0	100.0	95.7	87.5	83.1	
1/2/3	0.0	4.3	12.5	17.0	
ER & HER2					*0.0016
ER -. HER2 -	13.3	8.7	8.7	35.9	
Others	86.7	91.3	91.3	64.1	
ER, HER2 & CK5/6					0.0923
ER -. HER2 -, CK5/6: 1/2/3	0.0	4.3	4.2	15.4	
Others	100.0	95.7	95.8	84.6	

Table 2 Statistical assessment of QSOX1 protein expression with molecular subtypes of breast cancer. QSOX1 expression was grouped into 4 categories based on the percentage of cells stained and the intensity of QSOX1 expression: 0: no expression, 11/12/21: weak, 22/13/31: moderate and 32/33/23: strong (see material and methods for detailed explanation). Each number represents the percentage of QSOX1 positive or negative cells within each molecular subtype of breast cancer (n=total number of tissue samples within each category). Pearson's chi-square test was performed to determine a relationship between the molecular subtypes and QSOX1 expression. Statistical significance using a 2-sided *P* value was set at $P \leq 0.05$*.

with lower grade tumors. This is consistent with an association between QSOX1 expression and more aggressive ER+ tumors. While we do not have intrinsic subtype subclassification, relapse or survival data for the TMA tumors, it will be of interest to determine whether the ER+ tumors with elevated QSOX1 have a high proliferative index (Ki67 immunostain), and exhibit a relatively low percentage level of Progesterone Receptor positivity, suggestive of a Luminal B tumor.

Evaluation of QSOX1 expression by Western blot

QSOX1 expression in human breast adenocarcinoma was assessed in 5 different breast tumor cell lines, and a transformed non-tumorigenic breast cell line, MCF10A (Blick, Widodo, Hugo, Waltham, Lenburg, Neve, and E. W. Thompson). Consistent with our previous studies in pancreas cancer (Katchman, Antwi, Hostetter, Demeure, Watanabe, Decker, Miller, Hoff, and Lake), the short form of QSOX1 is expressed as the predominant splice variant in each cell line examined (Figure 10C). Consistent with the GOBO and IHC expression data, we found that the expression of QSOX1-S protein was more highly expressed in luminal-like cell lines MCF7 (ER+), MDA-MB-468 (ER-) and MDA-MB-453 (ER-) compared to basal-like BT549 and MDA-MB-231 cell lines. Interestingly, QSOX1 was most weakly expressed in MCF10A which does not form tumors in immunodeficient animals.

Expression of QSOX 1 in tumor cells promotes cellular proliferation

To begin to assess the mechanistic role that QSOX1 plays in tumor cells we stably knocked-down QSOX1 expression in MCF7 and BT549 cells using two lentiviral shRNA constructs, sh742 and sh528 (Figure 11A). QSOX1 protein expression was assessed following stable knock-down relative to isogenic parental cell lines by western blotting. Densitometry of the QSOX1 protein indicates that sh742 and sh528 resulted in a knock-down of QSOX1-S expression in MCF7 cells by 85% and 82% respectively; for BT549 cells the knock-down was 65% and 77%, respectively (Figure 11A).

The growth rates of shQSOX1-transduced MCF7 and BT549 cells were then evaluated compared to isogenic controls (Figure 11B). We seeded an equal number of untransduced (parental), shScramble, sh742 and sh528 cells in 96 well plates and assayed for proliferation over 5 days using the MTT assay. ShQSOX1-transduced MCF7 and BT549 cells both displayed a decrease in cell growth compared to shScrambled and parental controls (Figure 11B). In MCF7, sh742 and sh528 showed a 66% decrease in cell growth, while sh742 and sh528 suppressed growth of BT549 by 78% and 69%, respectively by day 5. Microscopically, we did not observe any morphological changes associated with loss of QSOX1 expression in either cell line.

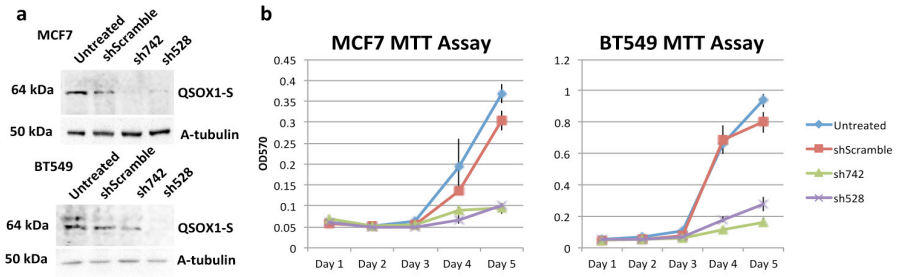


Figure 11. Reduced expression of QSOX1 leads to a significant decrease in tumor cell growth. MCF7 and BT549 breast tumor cell lines were transduced wtih lentiviral shRNA QSOX1 (sh742 and sh528). **a)** Western blots are shown using the same anti-QSOX1 polyclonal Ab as in figure 2 on cell lysates from MCF7 (percent decrease in sh742: 85% and sh528: 82%) and BT549 (percent decrease in sh742: 45% and sh528: 77%) cells. Western blots have been cropped and full images can be viewed in Supplementary Fig. S3. **(b)** MTT assay on MCF7 and BT549 cells transduced with shScramble, sh742 and sh528 assayed on Days 1 through 5. Percent decrease sh742 and sh528 day 5: 66%; percent decrease sh742 and sh528 on day5: 78% and 69%, respectively. Experiment was performed three times in triplicate; error bars represent SD from triplicate wells. Significance **, P= < 0.01.

Cell Cycle, Apoptosis and Autophagy Analysis

In non-tumor fibroblasts, expression of QSOX1 was correlated with the quiescent stage, G_o, of the cell cycle and overexpression of QSOX1 was shown to protect MCF7 cells for ROS mediated apoptosis (Morel et al.). This led us to hypothesize that a shQSOX1-mediated decrease in cell proliferation could be the result of abnormal regulation of the cell cycle, an increase in apoptosis or the result of autophagosome formation. To address this, propidium iodide (PI) was used in flow cytometry to evaluate the effects of shQSOX1 on cell cycle. In MCF7 cells, both shQSOX1 RNAs showed a slight decrease in G_1 and an increase (11-12%) in S phase, but neither shQSOX1 RNA sequence had any effect in BT549 cells compared to untreated and shScramble controls (Figure 12A & B).

Next we determined if the decrease in cellular proliferation was due to an increase in apoptosis or autophagy (Figure 12C & D). To assess apoptosis, we analyzed MCF7 transduced cells for Annexin V/ PI and BT549 transduced cells for increased expression of Caspase 3 (Plati, Bucur, and Khosravi-Far). We subsequently probed MCF7 and BT549 transduced cells for LC3, a protein that is necessary for auotphagosome formation (Chen and Klionsky). If the expression of QSOX1 prevented cellular apoptosis or autophagy we would expect to see an increase in expression of Annexin V, Caspase 3 and LC3 in shQSOX1 transduced cells, but we did not observe any changes (Figure 12A & B). This

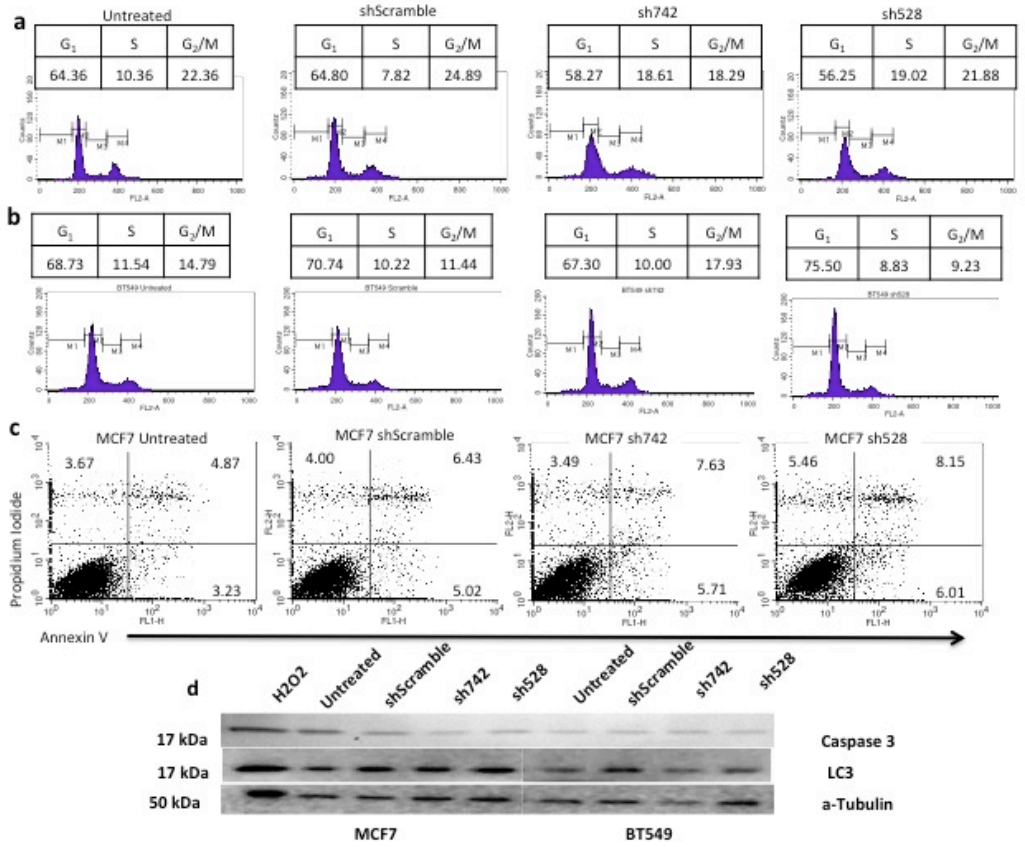


Figure 12. Suppression of QSOX1 in MCF7 and BT549 cells does not lead to an increase in apoptosis or autophagy. **a)** MCF7 and **b)** BT549 cells treated with shRNAs were analyzed for deviations in the cell cycle. Analysis was performed using propidium iodide to label DNA and analyze cells in G_1, S and G_2/M of the cell cycle by flow cytometry. Annexin V/ Propidium Iodide analysis was performed on **c)** MCF7 cells to assess apoptosis. Western blot analysis of **a)** MCF7 and BT549 untreated, shScramble, sh742 and sh528 total cellular protein probed for Caspase3 or LC3. BT549 cells incubated exogenously with 50uM H_2O_2 to induce expression of Caspase 3 (apoptosis) and LC3 (autophagy) is used as a positive control.

correlates with our previous results in pancreas cancer that the suppression of QSOX1 does not lead to cell death or autophagy.

Suppression QSOX1 expression inhibits tumor cell invasion

The process of tumor cell invasion involves the degradation of basement membrane components such as laminin, collagen and fibronectin before a tumor cell is able to invade other tissues (Bacac and Stamenkovic). We performed a modified Boyden chamber assay using Matrigel-coated inserts in which tumor cells must degrade the Matrigel and migrate through a membrane with 8um pores to gain access to nutrient rich media. Sh742 and sh528-transduced MCF-7 and BT549 tumor cells were added to Matrigel-coated, 8um pore inserts in serum-free medium. After 72 (MCF7) and 48 (BT549) hours of incubation, tumor cells that were able to degrade Matrigel and migrate through 8um pores onto the underside of the insert were counted (Figure 13A & B). Our results demonstrate that knockdown of QSOX1 expression in MCF7 leads to a 65% and 71% reduction in invasion of sh742 and sh528 transduced tumor cells, respectively. A 60% and 40% decrease in invasion through Matrigel for sh742 and sh528 BT549-transduced tumor cells was observed.

To prove that suppression of QSOX1 protein expression was responsible for loss of tumor cell invasion, we performed a rescue experiment in which we added exogenous recombinant human QSOX1 (rhQSOX1, generously provided by Dr. Colin Thorpe) to shQSOX1-MCF7

and shQSOX1-BT549. As a control for the enzymatically active QSOX1, a mutant rhQSOX1 in which the CxxC motif in the thiredoxin-1 domain was mutated to AxxA (rhQSOX1(AA), generously provided by Dr. Debbie Fass). Addition of enzymatically active rhQSOX1 rescued the invasive properties of both shQSOX1-MCF7 (Figure 13C) and shQSOX1-BT549 (Figure 13D) sh742 cells to the level of the shScramble control, while the addition of the rhQSOX1(AA) did not rescue invasion of tumor cells transduced with shQSOX1.

To determine if the decrease in the invasive capabilities of MCF7 and BT549 cells treated with sh742 and sh528 was due to an inability to degrade the matrigel allowing for the tumor cells to invade or if the loss of QSOX1 leads to a decrease in cellular motility. We performed an invasion assay using non-Matrigel coated PET membranes containing 8μm pore inserts. Both cell lines MCF7 and BT549 untreated, scramble, sh742 and sh528 transduced cells were seeded at equal densities in serum free media in the upper chamber. The inserts were then placed in wells containing 10% FBS in DMEM. After 24 hours of incubation, tumor cells that had migrated through 8μm pores onto the underside of the insert were counted (Figure 14A & B). Our results demonstrate that the loss of QSOX1 significantly affects the motility of MCF7 and BT549 cells. While the decrease in cellular motility (non-Matrigel) strongly suggests that QSOX1 plays a role in cellular movement we wanted to further determine

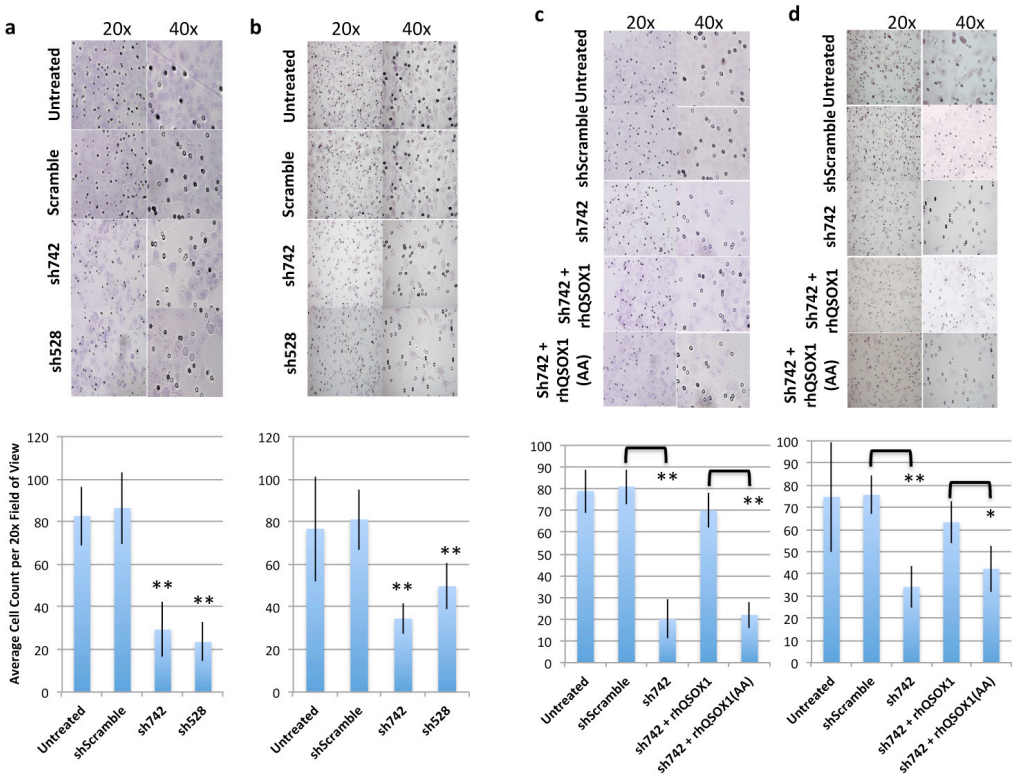


Figure 13. QSOX1 promotes tumor cell invasion in breast tumor cells. **a)** MCF7 and **b)** BT549 cells transduced with shSramble, sh742 and sh528 shRNAs were seeded at equal densities in the top chamber of Matrigel invasion wells and allowed to incubate for 48 (BT549) and 72 (MCF7) hours, after which cells that had digested Matrigel and migrated through the 8um pores were counted on the underside of the insert. Representative 20x and 40x images are presented. For MCF7 cells transduced with sh742 and sh528 there was a 65% and 71% decrease in invasion compared to shScramble controls, respectively. For BT549 cell transduced with sh742 and sh528 there was a 60% and 40% decrease in invasion. The invasive phenotype of shQSOX-transduced **c)** MCF7 and **d)** BT549 cells was rescued by exogenous incubation with catalytically active rhQSOX1. rhQSOX1 (AA) mutant is a mutant without enzymatic activity, generously provided by Dr. Debbie Fass. Graphs represent average ± SD (MCF7 and BT549 n=3), significance *, $P < 0.05$, ** $P < 0.005$.

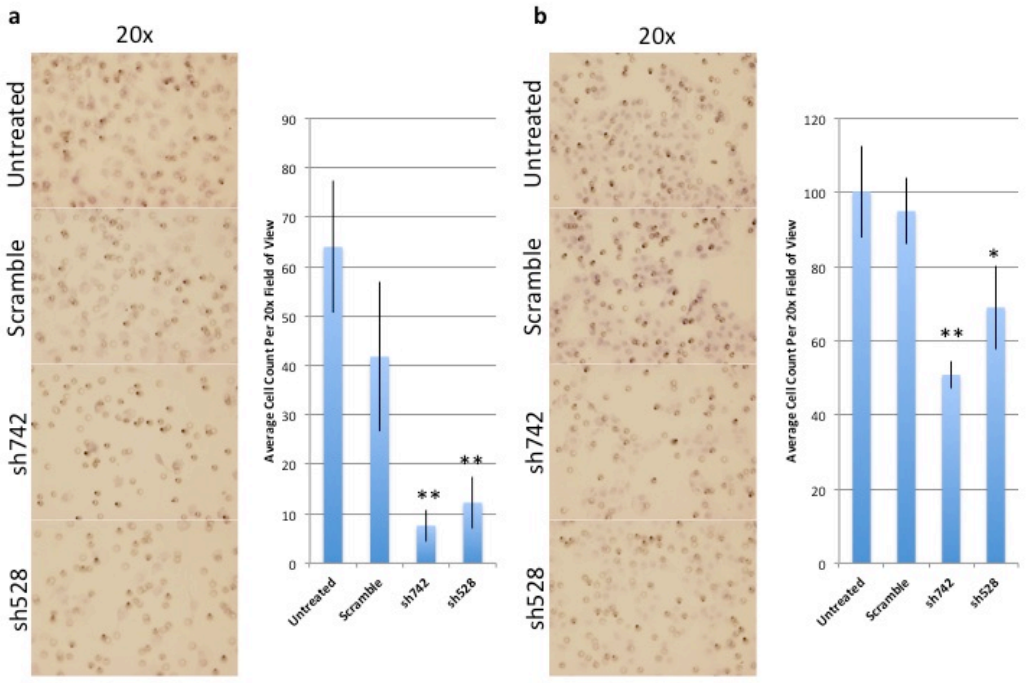


Figure 14. Reduced expression of QSOX1 in MCF7 and BT549 cells leads to a significant decrease in cellular invasion. A.) Untreated MCF7 and B.) Untreated BT549 cells were treated with Scramble, sh742 and sh528 shRNA's specific for QSOX1 and seeded in the top chamber of a non-coated invasion assay well and allowed to incubate for 24 hours. Representative 20x images are presented. In the MCF7 sh742 and sh528 treated cells there was a 44% and 38% decrease in cells that were able to invade to the underside of the membrane, respectfully. While in BT549 sh742 and sh528 cells there was a 75% and 62% decrease in cells that were able to invade through the membrane. Graphs represent average ± standard deviation (MCF7 n = 3; BT549 n = 3), significance *, $P < 0.05$, **, $P < 0.005$.

if the loss of QSOX1 in breast tumor cells also contributes to a decrease in protease function.

Decrease in QSOX1 leads to a decrease in Matrix metalloproteinase activity

Since knockdown of QSOX1 resulted in decreased breast tumor cell invasion, it was important to determine a mechanism. Matrix metalloproteinases (MMP) have been shown to play key roles in breast tumor invasion and metastasis (E. S. Radisky and D. C. Radisky). Both MMP-2 and -9 mRNA and protein levels have been shown to contribute to breast tumor invasion, metastasis and angiogenesis (E. S. Radisky and D. C. Radisky). Since previous work demonstrated that QSOX1-S is secreted into the extracellular matrix where MMPs are activated, we hypothesized that QSOX1 might help activate MMP-2 and -9 proteins. MCF7 and BT549 cells transduced with shScramble, sh742 and sh528 were plated at equal densities and allowed to incubate in serum free media for 48 hours, after which the supernatants were collected and analyzed by gelatin zymography to determine if the loss of QSOX1 leads to a decrease in the functional activity of MMP-2 and -9.

Initial analysis of the results indicate that MCF7 and BT549 possess similar MMP profiles even though it is known that BT549 cells are more invasive. Both MCF7 and BT549 supernatants contain MMP-9

homodimer (130kDa), a large amount of proteolytically active pro-MMP-9 (92kDa) with lesser concentrations of pro-MMP-2 (72kDa).

We found that supernatants from MCF7 cells transduced with sh742 and sh528 showed a 70% and 77% decrease, respectively, in pro-MMP9 activity compared to shScramble (Figure 15A). Supernatants from BT549 cells transduced with sh742 and sh528 showed a 34% and 88% decrease, respectively, in pro-MMP-9 (Figure 15B). Decreases in the proteolytic activity of MMP-9, using gelatin as a substrate, provide a mechanism for the shQSOX1-mediated suppression of invasion through Matrigel.

To extend our hypothesis that QSOX1 is activating MMPs post-translationally, we performed a Western blot on MCF7 and BT549 total cellular lysate as well as quantitative real time PCR (qPCR) to determine if the loss of QSOX1 affected MMP protein and RNA levels (Figure 15C and D). Our results indicate that the secreted amount of MMP-2 and -9 protein is similar between the untreated, shScramble, sh742 and sh528 samples in both MCF7 and BT549 cells (Figure 15C). Figure 15D demonstrates that the loss of QSOX1 also has no significant effect on the transcription of MMP-2 and -9. These results add confidence to our hypothesis that QSOX1 post-translationally activates MMPs.

Conclusion

In this study we show, for the first time that QSOX1 over-expression is associated with features of poor prognosis in breast cancer patients whose tumors express QSOX1. We also demonstrate the prognostic power of QSOX1 at the RNA and protein level. Analysis using GOBO of over 1800 breast cancer patients revealed that QSOX1 expression in luminal B breast cancer correlates with very poor RFS and OS (Figure 9C and D). Additionally, expression of QSOX1 mRNA in the GOBO analysis and IHC correlate with higher-grade tumors in our breast tumor TMA analysis (Table 2). Importantly, in patients who did not receive systemic therapy (presumably due to diagnosis of very early stage disease), QSOX1 appears to be a predictor of poor OS (Table 1). These data collectively suggest that QSOX1 is associated with bad-acting ER+ tumors and warrants further preclinical and prospective validation as a prognostic biomarker of ER+ tumors.

Our *in vitro* data suggest that QSOX1 has a conserved function in tumor cells regardless of the primary cell source. Tumor cells in which QSOX1 expression was suppressed using shRNAs grew at less than half the rate of shScramble and untreated controls in both MCF7 and BT549 cells (Figure 11B). While QSOX1 does not appear to play a role in cell cycle regulation, apoptosis or autophagy the loss of QSOX1 led to a dramatic decrease in both MCF7 and BT549 cells ability to degrade Matrigel and invade into a nutrient rich medium (Figure 13A and B).

Expression of QSOX1 promotes breast tumor growth and invasion *in vitro,* mediated mechanistically by post-translational activation of MMP-9 functional activity (Figure 15A and B). While further research is still needed to understand the role of QSOX1 *in vivo* the results presented here strongly suggest that targeted inhibition of QSOX1 may stall cancer progression.

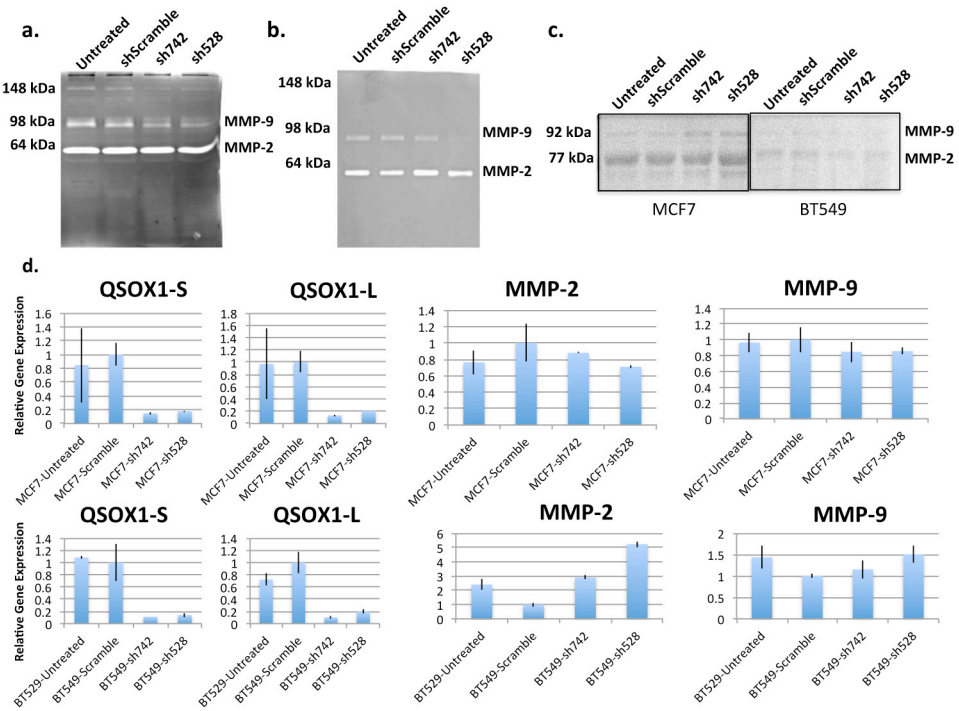


Figure 15. Reduced expression of QSOX1 in MCF7 and BT549 cells leads to a decrease in functional MMP-9 activity. Gelatin zymography of **a)** MCF7 and **b)** BT549 conditioned media shows a decrease in MMP-9 homodimers (130 kDa) and MMP-9 (92 kDa). The percent decrease in MMP-9 expression in MCF7 was: sh742: 70%; sh528: 77%, and in BT549 was: sh742: 34%; sh528: 88% compared to shScramble control. **c)** Western blots of total cellular lysate from shRNA treated MCF7 and BT549 probing for MMP-2 and -9. Percent decrease of MCF7 (sh742: 26%; sh528: 20%) and BT549 (sh742: 26% ; sh528: 78%) compared to shScramble control. Full images can be seen in Supplementary Fig. S3. **d)** QPCR of QSOX1 transcripts and MMP-2 and -9 transcripts. The graph represents relative gene expression calculated as $\Delta\Delta C_q$ using *GAPDH* as the endogenous reference gene.

CHAPTER 5

DISCUSSION AND CONCLUSION

The discovery and subsequent study of factors that contribute to tumor cell growth and invasion provide an opportunity to develop targeted therapeutics that could be used alone or in combination with other anti-neoplastic agents. The ability to effectively treat and cure patients with advanced metastatic tumors will be the turning point in cancer therapy. Prior to our discovery of a short peptide secreted in patient plasma, it was not previously known that QSOX1 was over-expressed in pancreatic tumors (Antwi, Hostetter, Demeure, Katchman, Decker, Ruiz, Sielaff, Koep, and Lake). The results presented within this thesis suggest that QSOX1 is a commonly over-expressed protein in PDA as well as breast adenocarcinoma making it a potential therapeutic and diagnostic target. To extend these initial findings we began to investigate why pancreatic and breast tumors over-express QSOX1, and mechanistically, what advantage it affords tumors.

To determine if QSOX1 was also over-expressed in breast cancer, a GOBO analysis was performed using data from over 1800 breast cancer cases (Ringnér, Fredlund, Häkkinen, Borg, and Staaf). GOBO is an open source software program that allows one to analyze the expression of specific genes from over 1800 breast tumor samples and 51 cell lines using data generated with Affimetrix U133A microarrays. We correlated

our GOBO analysis data with IHC analysis of tumor tissue samples probed for QSOX1, ER +/-, HER2, and CK5/6 (Table 2). We found a very strong correlation between the RNA expression of QSOX1 found in the GOBO analysis and our IHC results from breast TMAs. A prominent finding in this analysis is that the highest levels of QSOX1 expression in luminal B breast cancer correlate with very poor RFS and OS (Figure 9C & D), the median survival in patients with luminal B breast cancer who over-express QSOX1 is ~4 years. The prognostic power of QSOX1 expression for RFS and OS increases when luminal B breast cancer cases are divided into quintiles using the GOBO analysis tool for which patients with the highest fifth expression of QSOX1 have RFS of less than 2 years and OS of less than 3 years. Additionally, expression of QSOX1 mRNA in the GOBO analysis and protein in IHC correlate with luminal tumors and higher-grade tumors in our breast tumor TMA analyses (Table 1 and 2). Expression of QSOX1 did not correlate with survival in HER2 enriched tumors, ER- tumors, or in tumors subtyped as basal-like. Importantly, in patients who did not receive systemic therapy (presumably due to diagnosis of very early stage disease), QSOX1 appears to be a predictor of poor OS (Table 1). However, this association was not strong until more than 5 years post diagnosis. Collectively this suggests that QSOX1 is associated with bad-acting ER+ and further analysis will also need to be done to correlate QSOX1 protein expression with OS and RFS in luminal B tumors using Ki-67 as a marker of proliferation.

To determine what advantages QSOX1 provided to tumor cells we inhibited QSOX1 using shRNAs. The shRNAs specific to QSOX1 were packaged into lentivirus allowing us to generate stable pancreatic and breast tumor cell lines. The application of lentiviral transduction of shRNAs over transient tranfection of siRNA/shRNA allowed us to improve our percent knock-down of QSOX1 as well as providing us with highly reproducible results. As discussed in the introduction one of the first hallmarks of cancer is the ability of a tumor cell to proliferate uncontrollably. Tumor cells, both pancreatic and breast cells, in which QSOX1 protein expression was suppressed by shQSOX1 grew more slowly over time than the shScrambled and untreated controls as measured by an MTT assay (Figure 3C and 11B). We evaluated the growth of both pancreatic and breast tumor cells over a five day period and observed greater than 50% decrease in cell growth in all cell lines tested to date.

Our attempt to explain the decrease in cell growth as an alteration in cell cycle regulation, increase in apoptosis or autophagy failed to demonstrate changes that could explain our MTT results (Figure 4, 5, and 12). Contrary to previous statements implicating QSOX1 as a cell cycle regulator (Coppock, Cina-Poppe, and Gilleran), our results suggest that while the loss of QSOX1 in Panc-1 and MCF7 (luminal-like) cells shows a minor, but consistent stall in G_1, BxPC3 and BT549 (basal-like) cells do not follow this cell cycle example. This suggests that the role of QSOX1 in

cell cycle regulation could be cell type and tumor stage dependent (Figure 4 and 12). Combined, these results from two different tumor types suggest that cell cycle is not an important feature in the tumor biology of QSOX1.

Early experiments by Donald Coppock found that QSOX1 is secreted from WI-38 lung fibroblast cells as the cells become confluent and entered into a quiescent state. These experiments suggested that QSOX1 was involved in the transition from G_0 to G_1 in the cell cycle although this hypothesis was never validated (Coppock, Cina-Poppe, and Gilleran). While our experiments suggest that in specific tumor types QSOX1 may influence the cell cycle regulation they do not suggest that QSOX1 is an essential protein involved in transitioning cells from one stage in the cell cycle to the next. Another factor comparing early QSOX1 experiments with our results is cell type. WI-38 cells are non-tumor lung fibroblasts whereas our work was done with human malignant tumor cell lines.

Our analysis of apoptosis and autophagy as a second possible mechanism contributing to the observed decrease in cell growth did not reveal significant increases in Annexin V/ propidium iodide, Caspase 3 or LC3 (marker for autophagosome formation). In both pancreatic and breast tumor cells we failed to show significant increases in annexin V/ propidium iodide double positive cells as well as caspase 3 expression (BxPC3 - 8%; Panc-1 – 1.5%; MCF7 – 2%; BT549 – no increase in Caspase 3) (Figure 5 and 12). At this time, it is not known why or how QSOX1 drives growth of

tumor cells. However, the discovery that the loss of QSOX1 leads to a decrease in MMP activity could provide a possible explanation as to our observed decrease in growth. As MMPs cleave BM components they release growth factors, cytokines and chemokines that bind to receptor tyrosine kinases and promote cell growth. Within the same context the cleavage of different BM proteins also provides binding sites for integrin receptors that drive growth as well as promote cellular motility and invasion (E. S. Radisky and D. C. Radisky). Thus the loss in MMP functional activity could lead to a decrease in the availability of growth factors providing a possible explanation for the consistent decrease in cell growth observed in all of the cell lines tested.

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 (Fass). Currently 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 other sulfhydryl oxidases could help explain why the loss of QSOX1 does not lead to significant alterations in the cell cycle and apoptosis.

Because QSOX1 appears to be involved in tumor cell proliferation and to some extent, viability, the activity of QSOX1 in tumor cells is certainly not limited to post-translational activation of MMPs. QSOX1 may play a role in many cellular functions such as formation and maintenance of the ECM. Microscopic examination of shQSOX1-transduced tumor cells compared to shScrambled-transduced tumor cells demonstrated more detached and rounded cells in the shQSOX1 transduced cells.

Furthermore preliminary data from The Fass lab at The Weizmann Institute suggest that loss of QSOX1 significantly alters the expression and secretion of basement membrane proteins such as lamanin (Personal Communication). Basement membrane proteins, lamanin and collagen, play a significant role in influencing cell proliferation through their interaction with integrin receptors. One explanation for our observed decrease in cellular proliferation could stem from the lack of paracrine interactions via integrin receptors with lamanin and collagen that stimulate cell growth (Albini and Sporn; Shattil, Kim, and Ginsberg).

Another hallmark of cancer is invasion (Hanahan and Weinberg). Previous work by Portes et al. showed that QSOX1 is ubiquitously expressed in rat embryos eventually localizing to the testis a few days after birth. Numerous proteins involved in epithelial-to-mesenchymal transition that are expressed during development are reactivated during tumor progression contributing to tumor cell migration (Katz et al.; Chaffer and Weinberg; Maier, Wirth, and Beug). We hypothesized that the over-

expression of QSOX1 in pancreatic and breast 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 pancreatic and breast tumor cells to invade through Matrigel and non-Matrigel coated inserts *in vitro* (Figure 6, 7, 13 and 14).

Initially our results in both BxPC3 and Panc-1 cells led us to hypothesize that QSOX1 is a marker of highly aggressive tumors and therefore would not be expressed as strongly in cell lines that are poorly invasive. MCF7 cells are a poorly invasive luminal-like breast cancer cell line, while BT549 cells are a basal-like highly invasive cell line (Blick, Widodo, Hugo, Waltham, Lenburg, Neve, and E. W. Thompson). Surprisingly we discovered that QSOX1 expression is higher in MCF7 and other luminal-like cell lines compared to BT549 and other basal-like cell lines (Figure 10). Although the invasive capabilities are dramatically different between these two cell lines, QSOX1 knock-down suppressed growth and invasion in both cell lines. This finding indicates that the advantage QSOX1 provides to breast and pancreas tumors may be universal among phenotypically different tumors of different histological origin. However, one cannot draw this definitive conclusion from the behavior of cells cultured in 2D (Martin et al.; Rizki et al.). The relevance of QSOX1 is likely to become functionally relevant when considered not only in specific molecular subcontext (such as ER+ tumor cells), but in

specific environmental contexts within the 3D pancreatic and breast tumor microenvoronment with the full complement and complex interplay of autocrine and paracrine signaling components known to be important in tumor progression (Polyak; Michor and Polyak; Hu and Polyak).

While our results show that the loss of QSOX1 affects tumor cell motility, the loss of motility does not completely explain the dramatic decrease in the cells ability to degrade Matrigel suggesting that there are compensatory mechanisms contributing to overall decrease in the invasive abilities of the tumor cells. These results demonstrate that there are clear differences between the capacities of BxPC3, Panc-1, MCF7 and BT549 to degrade basement membrane components and invade through the membrane. This could be due to a myriad of factors such as other proteases secreted, the stage and subtype of the tumor and genetic differences between the cell lines (Deer et al.). To determine if this reasoning was correct, we performed gelatin zymography as a way to analyze the matrix metalloproteinase activity. There are 23 known human MMPs as well as 4 known tissue inhibitors of MMPs (TIMP) that aid in regulating the expression and activation of these proteolytic enzymes (Tallant, Marrero, and Gomis-Rüth). MMPs are zinc-dependent proteolytic enzymes that degrade ECM components (Kessenbrock, Plaks, and Werb). The expression patterns of MMPs are variable depending on tumor type, and even individual cell line.

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, Panc-1, MCF7 and BT549 cells differ as seen in figure 8A, B and 15A, B, we found that suppression of QSOX1 leads to a decrease in pro-MMP-2 and -9 activity.

In pancreatic and breast cancer the majority of MMPs are secreted in their inactive form and activated extracellularly (Tallant, Marrero, and Gomis-Rüth; Bauvois). Activation of MMPs occurs through the release of a covalent Cys^{73}–Zn^{2+} bond ("Cysteine Switch") and through cleavage and activation by plasmin, serine proteases, and other MMPs or TIMPs as well as interactions with basement membrane components such as lamanin, collagen and fibronectin (Tallant, Marrero, and Gomis-Rüth; Kessenbrock, Plaks, and Werb; E. S. Radisky and D. C. Radisky). It is possible that QSOX1 helps to re-fold MMPs after the cysteine switch and cleavage of pro-domain. Thiol binding proteins such as glutathione have been shown to help fold and activate MMPs (Kessenbrock, Plaks, and Werb). MMP-2 and -9 play an important role in pancreatic and breast cancer progression with 93% of tumors expressing MMP-2 compared to normal tissue (Bloomston, Zervos, and Rosemurgy; Köhrmann et al.). While reports

implicating MMP-9 in the progression of pancreatic cancer are limited, Tian reported the proteomic identification of MMP-9 in pancreatic juice from patients with pancreatic ductal adenocarcinoma (Tian et al.). Pryczynicz et al. also found a relationship between MMP-9 expression and lymph node metastases (Pryczynicz et al.). Numerous reports implicate MMP-2 as a prominent protease responsible for pancreatic tumor metastasis (Bloomston, Zervos, and Rosemurgy; Köhrmann et al.; Bauvois).

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 (Snoek-van Beurden and den Hoff; Tallant, Marrero, and Gomis-Rüth). A limitation of the zymography shown here is that it is limited to MMPs whose substrate is gelatin. It is possible that QSOX1 is involved in activation of other MMPs with different substrates as well as any one of the other proteases involved in degradation of basement membrane components. This may be investigated in future work.

Following up on our initial hypothesis regarding MMP activation by QSOX1 we performed a western blot analysis on total cellular lysate from MCF7, BT549, BxPC3 and Panc-1 shRNA treated cell lines. Our result revealed that the overall levels of secreted MMP-2 and -9 are nearly equal among the untreated, shScramble and shQSOX1 treated samples leading us to further hypothesize that QSOX1 is involved in the proper folding of

MMPs before they are secreted and that the loss of QSOX1 leads to proteolytically inactive MMPs as shown in figure 8 and 15. To further confirm our conclusions that MMP activation is a post-translational event, we performed qPCR on BxPC3, Panc-1, MCF7 and BT549 shQSOX1 treated cells. 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 in BxPC3, Panc-1 and BT549 cells while MCF7 showed a slight decrease in MMP-2 and -9 expression compared to our shScrambled control. 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.

It is not known if QSOX1 aids in the proper folding of MMPs intracellularly or extracellularly, 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-S activates them in the extracellular environment. To further understand this interaction it would be useful to determine the preferred pattern of substrates for QSOX1.

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 MMP activity through a feedback loop (Wu). Although we hypothesize that QSOX1 may activate MMPs directly by involvement in the cysteine switch activation mechanism (Snoek-van Beurden and den Hoff; Bloomston, Zervos, and Rosemurgy), ROS produced by QSOX1 may be indirectly activating MMPs, as MMP activation has been reported to depend on an oxidative environment (Wu).

Our results underscore the need to further understand the role that QSOX1 plays in tumor and normal cells, and how at the molecular level, it activates MMPs, helps drive tumor cell growth and contributes to cell motility. This information will be useful during development of inhibitors of QSOX1 that may work upstream of individual MMPs. QSOX1 is expressed during embryonic development in mouse and rat during key migratory stages (Andrade, Stolf, Debbas, Rosa, Kalil, Coelho, and Laurindo). This developmental data combined with our results indicating that QSOX1 expression facilitates degradation of basement membranes suggests that tumor cells over-express QSOX1 to allow them to degrade basement membranes and invade into adjacent tissues or into circulation.

QSOX1 expression in luminal B breast cancer subtype may help further stratify which tumors are likely to be more aggressive, leading to poor overall survival. If confirmed by other groups, our finding that QSOX1 is a predictor of poor OS and RFS in luminal types of breast cancer may provide another tool for physicians and their patients to decide

whether to more aggressively treat patients whose tumors express high levels of QSOX1.

The role of QSOX1 in cancer progression is only now beginning to be understood. Within this thesis we have shown that QSOX1, a novel protein in the field of cancer biology, is involved in several steps in the hallmark of cancer. Future studies determining the substrates that QSOX1 interacts with will not only aid in our basic understanding of tumor biology but will also allow us to combine anti-neoplastic agents with QSOX1 inhibitors providing us with the opportunity to slow the growth and invasive potential of tumor cells.

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