INHIBITION OF THE GLUTAREDOXIN SYSTEM INCREASES DOXORUBICIN SENSITIVITY IN HEPATOCELLULAR CARCINOMA BY IMPAIRING THE NRF2-DEPENDENT ANTIOXIDANT RESPONSE

THESIS

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Christy Xavier, B.S.

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SHORTHAND NOTATIONS

HCC = hepatocellular carcinoma
Grxs = glutaredoxin system (Grx1 and Grx2)
GSH = glutathione
GR = glutaredoxin reductase
Nrf2 = Nuclear erythroid-like factor 2
Keap1 = Kelch-like ECH-associated protein 1
NQO1 = NADPH quinone 1
HO-1 = heme oxygenase 1
Trx1 = thioredoxin 1
Trx2 = thioredoxin 2
SOD = superoxide dismutase
GST = glutathione-S-transferase
ROS = reactive oxygen species
DOX = doxorubicin
Grx1-sh = Grx1 shRNA
Grx2-sh = Grx2 shRNA
Scramble-sh = Scramble shRNA
DMEM = Dulbeco's Modified Eagle Medium
PBS = Phosphate Buffer Saline
FBS = fetal bovine serum

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Hepatocellular Carcinoma

Hepatocellular carcinoma, commonly referred to as malignant hepatoma, is the most common type of liver cancer and is the third leading cause of cancer-related deaths [1]. It kills approximately 1.25 million people worldwide each year and accounts for 1.5% of cancer worldwide [2]. It usually arises as a complication from diseases like primary viral hepatitis infections or cirrhosis. Unvaccinated individuals of Hepatitis B and C were the primary candidates for hepatocellular carcinoma in Eastern Asia, but new studies have discovered more risks, and the number of hepatocellular carcinoma patients are slowly increasing despite the increase in vaccinated individuals [2]. These risks include age, alcoholism, aflatoxin exposure, autoimmune disease, chronic liver inflammation, Wilson's disease and Type II diabetes [2]. Men tend to be at a higher risk than women to get hepatocellular carcinoma. Because of these risks, hepatocellular carcinoma is becoming a more prominent and difficult problem in the United States.

Hepatocellular carcinoma usually assumes three types of macroscopic pathologic forms. It can grow to be nodular or multifocal, massive or unifocal, or diffusively infiltrative. Microscopically, it can be classified in four different ways: trabecular, pseudoglandular, solid and scirrhous [3].

Traditional treatments include surgery, radiation therapy, and chemotherapy. However, surgery is only successful in 10-20% of the patients due to the malignancy, late diagnosis, and drug resistance especially [2]. If the tumor is not removed, patients can only expect another three to six months to live. Chemotherapy usually follows surgery for smaller tumors. Radiation therapy is another option, but due to accompanying cirrhosis and other liver diseases that occur with hepatocellular carcinoma, it becomes increasingly difficult to use radiation therapy [1]. Usually, in advanced cases, patients must rely on a liver transplant. With advancements in many drugs,

hepatocellular carcinoma is more manageable, but drug resistance is unfortunately becoming more prominent in today's society.

Doxorubicin and Cancer Drug Resistance

Doxorubicin is used as a common anti-cancer drug often used in conjunction with other anti-cancer therapies. It often slows or stops the growth of cancer cells. It is derived from an anthracycline antibiotic and has been researched for multiple uses other than cancer therapy [4]. There are currently over 2000 derivatives of doxorubicin currently on the market and used in research. It is administrated as a hydrochloride salt intravenously in cancer patients.

It is well known for its strong effectiveness especially due to its mechanism of action. Doxorubicin binds and intercalates with DNA, often preventing macromolecule synthesis. The planar aromatic chromophore within the molecule intercalates between two base pairs, while the six-membered daunosamine sugar positions itself within the minor groove [5]. The sugar specifically interacts with neighboring base pairs on the two different sides of the intercalation site. Due to the intercalation, topoisomerase II cannot perform its job of relaxing the highly negatively charged supercoils of DNA during transcription. After it breaks the DNA for replication, doxorubicin then binds and stabilizes the topoisomerase II complex [5]. This completely prevents the DNA from resealing and ultimately stops replication. Histones can also be affected by being removed due to intercalation. As such, DNA damage response, epigenome and transcriptome are deregulated. Without replication, the cell can no longer grow and will usually die.

Doxorubicin especially is important in the fact that it can generate cytotoxicity due to free radical production. The mechanism behind doxorubicin's free radical generation is still relatively

controversial. However, two methods have been proposed. Considering that doxorubicin targets topoisomerase II, doxorubicin has been proposed to induce hydrogen peroxide indirectly [5]. The hydrogen peroxide production is said to be a side byproduct of binding to topoisomerase II. Eventually, this oxidative DNA damage causes PARP and NAD(P)H oxidase activation, leading to an increased mitochondrial membrane potential [5]. It is also reported to cause caspase-3 activation. Secondly, doxorubicin can stimulate the ryanodine receptor and open this receptor, so that intracellular calcium increases [6]. This induces calcium-dependent reactive oxygen species (ROS) generation and can lead to overwhelming oxidative stress. Free radicals are usually taken care of by certain antioxidant enzymes, but usually, an abundance of oxidative stress can lead to cell death [7].

Multiple cancers however can develop resistance to premier anti-cancer drugs such as doxorubicin. Time is often a determining factor of drug resistance. Most cancer patients face a long-term medicine regiment. Because of this, cancer cells can easily develop mechanisms to thwart the effectiveness of various anti-cancer drugs, regardless of differences in structure. This includes efflux pumps, decreased uptake proteins, manipulation of the apoptotic pathway, and increased antioxidant enzyme expression [7,8]. Manipulation of multiple pathways as well as novel mutations developed over time have contributed to anti-cancer drug resistance. Specifically, variations in the apoptotic pathway like overexpression of Bcl2 or inhibition of Bax and overexpression of DNA repair enzymes all confer drug resistance. This can really impair doxorubicin's apoptotic ability since doxorubicin is also known to functionally decrease Bcl2 and increase Bax expression [9]. Because doxorubicin is a ROS-inducing drug, doxorubicin toxicity is often thwarted by the upregulation of multiple antioxidant enzymes that counteract ROS generation. Although doxorubicin has implications in suppressing catalase activity, the

overexpression of several antioxidant enzymes has overpowered doxorubicin's effectiveness, leading to decreased doxorubicin sensitivity [5]. Because of this, oncologists face a difficult problem in increasing the doxorubicin administered to cancer patients, but this has only caused problems in toxicity and abundant oxidative stress [8,9]. Moreover, questions arise about doxorubicin's ability to discriminate between healthy and cancerous tissue. However, several studies support that doxorubicin's activation of the p53 pathway following caspase-3 activation is much more preferred in cancerous tissues than neighboring healthy tissue [10]. Nonetheless, doxorubicin's capabilities to induce oxidative stress and apoptosis may be detrimental to normal cells if higher doses are administered due to doxorubicin resistance, leading to severe side effects and increased patient death.

Most methods involving drug resistance are not very discriminatory, causing multi-drug resistance. Multi-drug resistance limits the available number of effective drugs on the market, making it even more difficult to treat cancer patients. Without discovering a drug target than can cause both the death of cancer cells and sensitize cancer cells to anti-cancer drugs, cancer patient death will continue to increase.

Protein Oxidation and Oxidative Stress

Doxorubicin is unique in its ability to create ROS. An increase in ROS to the point where it overcomes the defense system or the antioxidant system causes oxidative stress, which can eventually lead to cell death. Oxidative stress can affect a variety of molecules such as lipids, DNA, and proteins [9]. In particular, cellular proteins are exposed to oxidative damage by chemical and natural means that may alter protein function and structure on a daily basis. Protein oxidation via reactive oxygen species (ROS) can be both beneficial and harmful. ROS formation

tends to be concentrated in the mitochondria and involve the oxidation of many enzymes in order to perform multiple functions within the body like insulin signaling and release, cell cycle regulation, and hypoxic response [11]. Mitochondria use small amounts of ROS to ultimately power a lot of their redox reactions and help with vital cellular functions. Abundant ROS though causes detrimental oxidative stress, which can lead to both protein and organ damage and death. So, it's important to reverse this process to allow for normal protein activity even in times of oxidative stress.

Protein Glutathionylation

S-glutathionylation is one of the primary means of protein oxidation and involves covalently linking tripeptide glutathione (GSH) to protein thiols by a disulfide bond [12]. Cysteine residues with open sulfhydryl groups are often the target of GSH addition, and unfortunately, cysteine tends to be located in active sites of proteins. The tripeptide, Glutamate-Cysteine-Glycine, is considered to be the most sensitive to oxidation [12]. GSH is found abundantly in many tissues but is often found localized to tissues most sensitive to oxidative stress such as the lens, red blood cells, brain, kidneys, and liver. Recent studies discovered that one percent of total glutathione is found conjugated with proteins in normal rat liver [13]. Glutathione level varies depending on cell type and intracellular conditions. There are independent pools of glutathione found in the nucleus, the mitochondria, and the endoplasmic reticulum and are specialized to cater to the functions of each organelle. Generally, in times of oxidative stress, GSH-conjugated proteins may be more abundant and can alter enzyme function. Protein glutathionylation occurs mainly both by direct oxidation or thiol-disulfide exchange and less frequently through S-nitrosothiols, sulfenic acids, or thiyl radical protein intermediates [12].

Glutathionylation of enzymes especially metabolic proteins often translate into inactivation or inhibition, but glutathionylation is more collectively called as a regulatory mechanism due to its ability to either inhibit, increase, stabilize, or destabilize enzymatic activity and alter DNA binding for transcription factors [13]. For example, carbonic anhydrase III phosphatase activity is increased with glutathionylation, and HIV protease glutathionylation increases stability of the protein. Even so, glutathionylation often correlates to an increased oxidative stress state of the cell, which has implications in disease and toxicology [13]. Recently, it has been discovered that glutathonylation may act as an inflammatory activator by glutathionylating peroxiredoxin 2 and other inflammatory molecules in times of oxidative stress [14]. However, most oxidative stress-induced glutathionylation can cause abundant protein damage and cell death. Thus, reversible glutathionylation is important in order to maintain protein function in oxidative damage, redox signaling, and prevent organ damage and death.

Glutaredoxin System

Reversible glutathionylation is mediated by the glutaredoxin system. Glutaredoxin or often referred to as thiotransferase system is composed of glutathione (GSH), glutaredoxin (Grx), and glutathione reductase (GR) [15]. Glutaredoxin functions as a protein thiol repair enzyme that cleaves protein glutathione (PSSG) using GSH as a cofactor. Glutaredoxin system has two distinct subsets, Grx1 and Grx2. Grx1 and Grx2 are both isoforms in this family that are found in mammalian cells. Grx1 is primarily localized to the cytoplasm, but recent findings have supported that Grx1 has functions in the intermembrane space of the mitochondria and nucleus [16, 17]. Found to be less active and in smaller amounts generally, Grx2 is rather located exclusively in the mitochondrial matrix, amounting to be 10 times more concentrated there than Grx1.

Approximately 18 kDa, Grx2 is slightly larger than Grx1 and has an important Cys-Ser-Tyr-Cys active site motif. The substitution of the serine in Grx2 for the proline in Grx1 allows for this high affinity [18]. Grx2 has three functional alternative splice variants – Grx2a, Grx2b, and Grx2c. Grx2a is ubiquitously expressed in the mitochondria, whereas Grx2b and Grx2c were thought to be restricted in the testes. New evidence shows that tumor cells also have numerous Grx2b and Grx2c transcripts that are possibly co-localized to both the cytoplasm and the nucleus [19].

Grxs Mechanism and Function

Protein structure for Grx is four beta sheets surrounded by three alpha helices. The key active site motif (Cys-X-X-Cys) is located between the first beta sheet and the first alpha helix in Grx1 and Grx2 [15, 18]. Grx1 and Grx2's strong reductant properties allows for selectively using either of the two cysteines found at either end of the motif to reduce certain kinds of disulfide bonds. Although there is support for monothiol and dithiol mechanisms, the monothiol mechanism is more accepted. In this process, the N-terminal active site cysteine is only necessary to complete deglutathionylation. Oxidized Grx can be reduced by 2 GSH to yield oxidized GSSG [15, 21]. The second GSH used to form another disulfide is the rate-limiting step in the reaction. Then, GSSG can be converted to its reduced form by glutaredoxin reductase with NADPH as an electron donor. The dithiol mechanism shows that Grx can use the C-terminal active site thiol as well to form a protein disulfide [22].

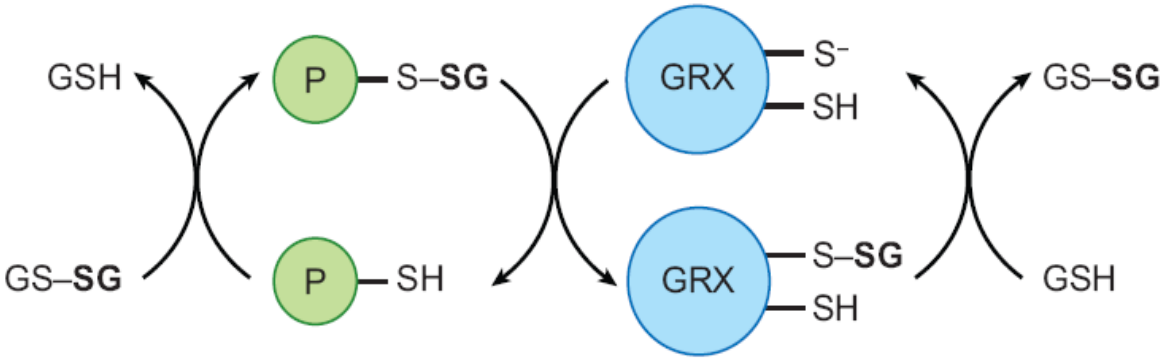


Fig 1. Glutaredoxin cleaves PSSG using GSH as a cofactor. Glutaredoxin reduces PSSG to P-SH using GSH as a cofactor and becomes oxidized itself. Glutaredoxin reductase then reduces glutaredoxin with the help of NADPH and GSH as a cofactor.

Grx1 has a unique GSH binding site located antiparallel to the Cys motif allows for a monothiol mechanism using NADPH as an electron donor [15]. Grx1 primarily exerts its reducing abilities mainly in the cytoplasm, which often links it to actin deglutathionylation and cell movement [20]. On the other hand, Grx2 in its inactive form is a dimer bound to Fe_2S_2 , but upon oxidative stress, triggers to an active monomeric state and deglutathionylates the target protein [18]. However, Grx1 remains a monomer in its inactive and active states.

Cellular-Mediated Protection via Grxs

Glutathione reversal is promoted by Grxs which protects the cell from extra damage induced by reactive oxygen species. Essentially, this process is activated depending on the delicate balance and ratio of [GSH]/[GSSG]. If this ratio decreases, the active site of Grx cannot be reduced, thus promoting the activity of Grx [22]. Grx exerts its protective effects and helps recovery of the cells. One of the primary functions first discovered for glutaredoxin was its ability to donate electrons to activate ribonucleotide reductase. Ribonucleotide reductase is a vital enzyme necessary for DNA synthesis and repair because it catalyzes the production of rNDP to dNDP [23]. Because

glutaredoxin primarily was discovered to share this function with thioredoxin, it was proposed that glutaredoxin was a back-up system for thioredoxin, but more evidence shows that despite that thioredoxin and glutaredoxin have homologous functions, glutaredoxin is heavily involved in other cellular protective functions. For example, Grx2 can be an extremely capable anti-apoptotic enzyme in the mitochondria, which is one of the most important features in relation to cancer. Since Grx2 is located exclusively in the mitochondria, a huge part of the electron transport chain relies on Grx2. Although majority of the electrons are used in ATP generation, a small percentage of the electrons are used to actually form reactive oxygen species which leads to cellular damage due to oxidation of complex I in mitochondria. Grx2 reverses the oxidation of complex I and restoring complex I to its active form [24, 25]. Increased Grx activity can repair damage caused by oxidative stress. Glutaredoxin is essential for the survival and protection of cells specifically because of its ability to also interact with the apoptotic pathway. Recent studies have found that glutaredoxin can directly upregulate Bcl2, an anti-apoptotic protein, and prevent caspase-3 activation [26, 27]. Glutaredoxin may also prevent cytochrome c release, one of the crucial steps in committing the cell to apoptosis [28]. Moreover, glutaredoxin has been implicated in the Akt pathway, a key survival pathway of the cell. Glutaredoxin can regulate Akt glutathionylation, which can lead to Akt activation and cellular protection [29]. Protective effects exerted by Grx ultimately leads to the survival of the cell.Grx functions are not limited to its strong reduction potential. Grx1 has been suggested to have smaller roles in the inner mitochondrial membrane and the nucleus. Grx1's Cys-27 residue in the active site as well as its core iron-sulfur cluster is noted to be required for its special redox sensing as well as peroxidase and transferase capabilities [22]. Grx1 has functions in reducing dehydroascorbate (Vitamin C), peroxiredoxins, and methionine sulfoxide reductase. Grx1 deficiency has often been linked to accumulation of superoxide anion

in yeast, showing the aptitude of Grx1 as a direct hydroperoxide scavenger and a vital antioxidant defense [30]. Grx1 is also involved in the detoxification of intracellular copper, increasing copper metabolism as needed [22, 31]. Grx1 may also be involved indirectly in antioxidant defense by restoring copper binding to Atox1 so that Atox1 can continue its antioxidant function and promote cell proliferation [31]. Grx1's cooperation with a lot of multiple antioxidant systems like thioredoxin directly and indirectly makes it a powerful antioxidant enzyme [26]. Like thioredoxin, the glutaredoxin family is involved in multiple alternative pathways in cellular functions such as the reduction of ribonucleotide reductase to allow DNA replication to proceed, the generation of reduced sulfur using 3-phosphoadenylylsulfate reductase, a signal transduction protein, and of course, in antioxidant defense [15, 26].

Grx2 may also have vital functions in converting toxic products to nontoxic metabolites. For example, dihydroascorbate (DHA) or the oxidized form of vitamin C can be reduced to ascorbate via GSH. Vitamin C can then catalyze the detoxification of reactive oxygen species to harmless metabolites such as water and oxygen. Working together with thioredoxin, it is very capable in transforming peroxide to two molecules of ROH and oxygen [26]. Grx2 also has its own unique abilities. For example, iron-sulfur clustering in the Grx2 center halo of Grx2's dimeric form is often deemed as the redox sensor. During oxidative insults, the redox sensor is activated and causes reduced clustering [32]. Diminished iron-sulfur clustering is often present in reduced forms of Grx2. Although less noted and explored, Grx2 may play a role in preventing the aggregation and formation of mutant forms of superoxide dismutase, both MnSOD and CuZnSOD [33]. It may also be an electron donor for mitochondrial 2-Cys peroxiredoxin (Prx3) like thioredoxin 2, which may also be involved in the redox signaling process [34]. Glutaredoxin 2 may also be involved in the degradation of oxidative proteins by regulating the 20S proteasome, the primary proteasome

involved in degrading cellular proteins. When glutaredoxin 2 deglutathionylates the 20S proteasome, chymotrypsin-like activity and post-acidic protein cleavage functions of the proteasome is recovered [35].

Increased oxidative stress leads to high amounts of GSSG and TR, and GSH is thus decreased. Grx1 and Grx2 are fairly flexible in receiving electrons from both GSH and thioredoxin (Trx) with accompanied NADPH oxidation to cause mitochondrial protection. Grx and Trx often cross-talk to increase the reliability of each system in performing its activities [26]. For example, Trx can reduce GSSG. Therefore, cells depleted in GSH can be once more abundant in GSH. In this case, Trx acts as a backup system [26].

Nrf2 Pathway

One of the crucial antioxidant pathways involved is the nuclear factor (erythroid-derived-2)-like 2 (Nrf2) pathway. Nrf2 is a 65 kDa molecule with a basic leucine zipper structure [36]. Normally, Nrf2 in its inactive state is kept in the cytoplasm bound to Keap1. With a half-life of only 20 minutes, Nrf2 is constantly targeted for ubiquitination by Keap1 and Cullin 3 with consequential degradation via the proteasome [37]. Of the seven functional domains located in Nrf2, Neh2 is equipped with seven lysine residues and is responsible for ubiquitin conjugation. The ETGE and DLG motifs in Nrf2 interact with Keap1, preventing ubiquitination and subsequent degradation [36]. When the cell is signaled to be in a particularly oxidative stress or electrophilic environment, oxidative stress usually acts upon Keap1's active site cysteine residues, preventing Keap1 from interacting with Nrf2 [38]. With the accumulation of Nrf2 in the cytoplasm, Nrf2 moves to the nucleus where it binds to the small Maf protein with the Neh1 functional domain and the antioxidant response element (ARE) [36]. Activation of ARE leads to the transcriptional

activation of several other antioxidant enzymes and proteins, like NADPH dehydrogenase (NQO1), heme oxygenase-1 (HO-1), glutaredoxin (Grx), thioredoxin (Trx), superoxide dismutase (SOD), catalase, glutathione peroxidase, and more [36, 37]. The ability of Nrf2 to regulate all these antioxidant enzymes often gives Nrf2 its name as the master antioxidant transcriptional regulator.

All these enzymes are considered vital antioxidants and distinguished by their ability to prevent oxidative damage and stress. NQO1 transforms enzymes and proteins back into their reduced state by the exchange of electrons between NADPH and NADP [38]. HO-1 may indirectly be involved in the antioxidant system by converting heme to other products such as iron (II), carbon monoxide, and biliverdin [38, 39]. The thioredoxin system are composed of protein thiol repair enzymes that specifically cleaves disulfide bonds. The thioredoxin family consists of the thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH and has many known implications when protecting the cell from oxidative stress. Together, Trx protein weighs about 12 kDa with 105 amino acids and a central motif Trp-Cys-Gly-Pro-Cys at the active site [15]. Thioredoxin has many isoforms with alternative splice variants that are widespread throughout the cytoplasm, nucleus, and mitochondria. Other antioxidant enzymes such as superoxide dismutase (SOD), catalase, and peroxidase to target oxidative products act upon specific ROS molecules and are often used in lesser amounts. Superoxide dismutase (SOD) can convert the cytotoxic superoxide radical (O_2^-) to oxygen or hydrogen peroxide [30]. Catalase then acts upon hydrogen peroxide to convert it to water and oxygen [40]. Glutathione peroxidase (GPx) is able to catalyze the reaction of hydrogen peroxide to water with the conversion of GSH to GSSG [37]. All these antioxidant enzymes work together to detoxify ROS and protect the cell.

In recent studies, Nrf2 is considered to also be overexpressed in cancer cells. Having Nrf2 readily available for transcription is essential for cancer cells to continue to survive [40]. Nrf2

overexpression allows for decreased ROS generation and increased apoptotic resistance, cell survival, proliferation, and tumorigenesis [41]. Although the survival-promoting effects of Nrf2 in cancer cells have been well defined, the redox regulation of Nrf2 pathway, particularly the post-translation modifications and the redox signaling of Nrf2, remains largely unknown.

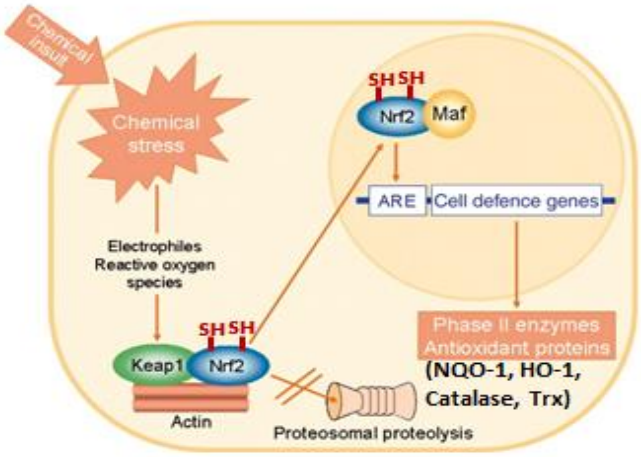


Fig 2. Nrf2 is the master transcriptional regulator of vital antioxidant enzymes. Nrf2 is normally kept in its inactive state bound to Keap1. However, chemical stress stimulated either by electrophiles or reactive oxygen species allows for the dissociation between Keap1 and Nrf2 by disrupting Nrf2's or Keap1's cysteine bonds. Nrf2 can then freely translocate to the nucleus, where it can bind ARE and Maf protein, which causes the transcription of phase II enzymes or antioxidant proteins such as NQO1, HO-1, catalase, and thioredoxin.

Cancer Redox Biology

Cancer redox biology is a relatively new field, but it is a largely promising field. There is an evident connection between the abnormal development and growth cellular processes that cause differentiation of cancer cells and the unusual electron flow in oxidation-reduction reactions in the cell that cause certain sensitive signaling pathways and gene expression. Reports support that malignant transformation and differentiation of cancer cells are often from abnormally carried out redox reactions [42].

Cancer often progresses by a prooxidant environment. Prolonged exposure to oxidants leads to activation of protein kinases and inactivation of protein phosphatases. Protein kinases often phosphorylate and activate transcription factors. Most of these transcription factors have redox regulatory sites often consisting of cysteine residues. Mutations that occur in these redox regulatory sites often lead to the conversion of proto-oncogenes to oncogenes that can lead to cancer development [42, 43]. Tumor suppressor genes are also inhibited in the process. Oxidants also promote cell growth signaling, as growth factors were activated in the presence of redox-sensitive ROS-mediated signaling pathways with involvement of certain oxidase enzymes associated with mitochondrial metabolism [44]. Metabolic ROS overproduction has also showed interesting results. This accompanied by mutations in mitochondrial electron transport chain proteins can elevate the possibility of differentiation of a normal cell to a cancerous cell. Differentiation of tumor cells is vital in determining not only prognosis but resistance to various standard anti-cancer therapies [36].

Cellular-mediated protection via many antioxidant enzymes is an unexpected boon for cancer cells though. Tumor cells often persist due to resistance to apoptosis and purposely increase antioxidant enzymes for further protection. Many reports support that thioredoxin system has a role in tumor growth and resistance [43, 45]. Previous studies using lung cancer cells in mice show that knock-down of TrxR1 in lung squamous cancer cells with overexpression of TrxR1 tend to be more sensitive to cancer drugs and anti-cancer therapies as well as have increased apoptosis of cancer cells [46]. Hence, TrxR1 and TrxR2 targeting drugs are now the determinants of successful cancer treatment.

Cancer Metastasis and Actin Glutathionylation

Metastasis can be linked to actin dynamics. This was first observed in neutrophils and how neutrophils travel to places following chemotaxis by purposely changing actin structure [47]. Actin has two distinct isoforms, F-actin and G-actin, which is strictly regulated by Grx1. F-actin or filamentous actin can easily polymerize and mobilize the cell. G-actin or globular actin is highly glutathionylated and cannot polymerize or mobilize the cell. Glutathionylation of beta-actin is said to occur via a spontaneous oxidation of a cysteinyl residue to a sulfenic acid with the intermediate GSH to form a mixed disulfide. Depending on the actin isoform, reactivity and oxidation to a protein thiol intermediary vary [35].

Grx1 deglutathionylates G-actin, the globular isoform, to F-actin in cancer cells, allowing for the mobilization of the cell. The deficiency or loss of Grx1 would cause the deregulation of actin, leading actin to be in a primarily G-actin state [35, 47]. Manipulation of actin remains another interest of anti-cancer therapy.

Grxs Knockdown as a Future Possibility for Anti-Cancer Therapy and Elimination of Resistance to Common Anti-Cancer Drugs

Cancer persists due to escape from apoptosis, and despite causing ROS overproduction, it takes advantage of antioxidant enzymes for continuous survival and protection [48]. Current therapies are showing to be futile, as resistance becomes increasingly possible [44]. Hence, combining molecular methods involving redox biology and drug therapy may all together slow down resistance and eliminate previously impossible tumors.

Previous studies have shown Grxs overexpression is commonly found in HeLa cells. HeLa cells subsequently are less sensitive to oxidative stress inducing drugs such as doxorubicin due to

reduced cytochrome c release [28]. Cytochrome c release to the cell surface is one of the first steps to signal a cell toward apoptosis. HeLa cells have also shown to manipulate redox-sensitive signaling in order to prevent caspase-3 activation by glutathionylating caspase-3 [27]. Caspase activation is also a crucial pathway associated with apoptosis and cell death. siRNA-treated HeLa cells specific to Grx2 showed to have increased sensitivity to phenylarsine oxide and doxorubicin as well as other oxidative stress inducing drugs [18].

Increasing evidence also surprisingly reveals that Grx2 is not exclusively found in the mitochondria of multiple cancer cells and testicular cells. It is rather found in abundant amounts in the cytoplasm as well [18]. Both Grxs are then known to be a reductant of oxidized Trx1-S2 and Trx2-S2 in the cytoplasm. Although TrxR is the matching and true reductant of Trx1 and Trx2, its highly sensitive active site selenocysteine can easily be inactivated by electrophilic compounds [16, 21]. Thus, the presence of reduced Trx1 and Trx2 in this case heavily depends on Grx2. Thus, overexpression of Grxs is involved in leading to better redox state in cancer cells, causing cancer survival and reducing the effectiveness of TrxR inhibitors [42, 46].

Taking advantage of altered metabolic states in cancer compared to normal cells can lead to the exploitation of cancer cells exclusively and selectively. Moreover, toxicity of chemotherapeutic agents as well as other drugs could be elevated if combined with the inhibition of antioxidant detoxification systems by targeting certain components such as Grx1, Grx2, Trx1, or Trx2. Antioxidants could also further protect other tissues from damage caused by chemotherapy and radiation therapy. Previous studies support the inhibition of MnSOD and catalase via adenovirus vector delivery to oral cancer cells can inhibit tumorigenesis [49]. MnSOD overexpression can lead to redox signaling aberrations and thus disrupt cell division. Glucose deprivation-induced metabolic oxidative stress is another characteristic of cancer cells often

manipulated. Increased glucose metabolism is compensated for by increased hydroperoxide production. Inhibitors of glucose and hydrogen peroxide metabolism has shown then to rather increase sensitivity to chemotherapeutic and radiation therapy drugs [50].

The theory of knocking down Grxs through RNA interference is a new proposed solution to combat cancer. Although the discovery of Grxs is relatively new and there is limited research available, Grxs has high potential due to its redox-signaling functions specifically in mitochondria and its elusive cross-talk with other redox systems such as Trx1 and Trx2. Grxs has been proven multiple times to be associated with cancer cell lines and be a primary reason for deviant cell differentiation, malignancy, and drug resistance [51].

Hypothesis and Rationale for Study

Cancer cells exhibit increased ROS generation and ATP demand that may promote cell proliferation, metastasis, cell survival and drug resistance through redox signaling [52]. This highlights the crucial role of ROS stress in tumor development and drug resistance. Targeting these biochemical properties of cancer cells with redox-modulating strategies is a feasible therapeutic approach that may enable therapeutic selectivity and to increase cancer cell sensitivity to doxorubicin [50]. However, the mechanism of redox regulation in the cancer cells is still very poorly understood. In particular, the function of a thiol-regulating enzyme, Grxs in liver cancer cells, is virtually unknown. More importantly, the cross-talk between Grx system and Nrf2, a powerful antioxidant transcription regulator, has never been studied. In previous studies, Nrf2 has been shown to be phosphorylated after sensing oxidative stress and thus promotes its translocation to the nucleus to upregulate antioxidant enzymes [53]. Similarly, previous studies have shown that reduced and unhindered cysteine residues are essential for oxidant/electrophile-sensing and

transcriptional activation of

Nrf2, indicating Nrf2's free thiols are critical for Nrf2 activation in response to oxidative stress [54]. However, Nrf2 regulation by S-glutathionylation remains elusive. Thus, it is hypothesized that knockdown of Grx1 and Grx2 sensitizes hepatocellular carcinoma cells to doxorubicin by impairing the Nrf2-dependent antioxidant response.

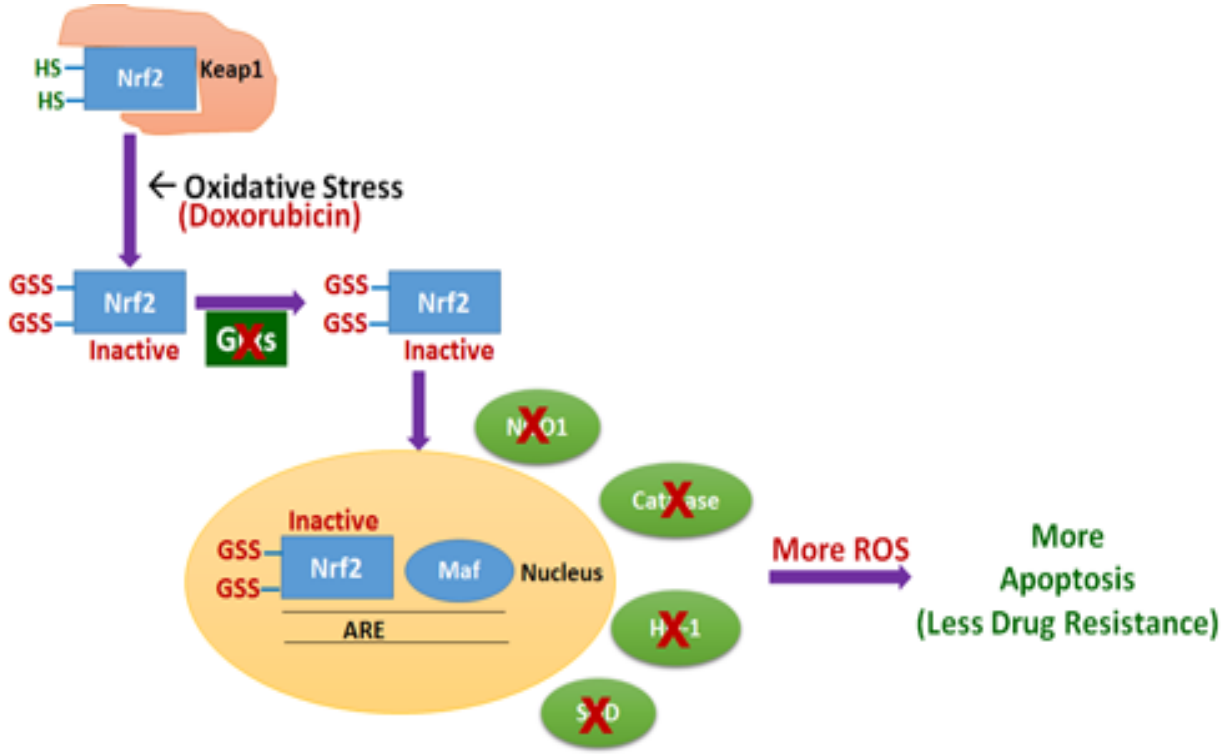


Fig 3. Glutaredoxin inhibition sensitizes hepatocellular carcinoma to doxorubicin by impairing the Nrf2-dependent antioxidant response. Oxidative stress caused by doxorubicin treatment causes the dissociation of Nrf2 from Keap1. Nrf2's cysteine residues becomes glutathionylated, causing Nrf2 to deactivate. Normally, since glutaredoxin is abundant in cancer cells, glutaredoxin can mediate the deglutathionylation of Nrf2. However, with glutaredoxin inhibition, Nrf2 remains in its inactive glutathionylated state. Because Nrf2's cysteine residues are hindered and Nrf2 remains inactive, Nrf2 will either get degraded in the cytoplasm by the proteasome or be unable to bind to ARE. This will ultimately attenuate the transcription of key antioxidant enzymes like NQO1, catalase, HO-1, and SOD. ROS overproduction causes oxidative stress that will overwhelm the cell, leading to more apoptosis and less doxorubicin resistance.

MATERIALS & METHODS

A. Preclinical Data Analysis of Grx1 and Grx2 Expression Levels in Hepatocellular Carcinoma

By collaborating with the 3rd Affiliated Hospital of Sun-Yat-Sen, we collected HCC tissue and the neighboring noncancerous tissue from five patients (two pairs of samples were analyzed in the preliminary study or cohort 1 in 2013 and the other three pairs were analyzed as cohort 2 in 2014). Protein was collected, loaded onto a 15% gel, and then blotted onto a PVDF membrane. The membrane was then tested for Grx1 and Grx2.

B. Culturing and Preparation of HepG2 Cells

HepG2 cell line (hepatocellular carcinoma) was obtained from Sigma-Aldrich. HepG2 cells were cultured in DMEM media supplemented with 1% penicillin and streptomycin and 20% FBS. Media was changed every 2-3 days and passed every week using trypsin.

C. shRNA-mediated Knockdown of Grx1 and Grx2 in HepG2

shRNA knockdown of Grx1 and Grx2 was done using the Santa Cruz Biotechnology protocol. HepG2 cells were seeded into a six well plate with added 1 ml of DMEM + 1% penicillin and streptomycin + 20% PBS and incubate cells overnight. Cells were grown for 24 hours to a confluency of about 90%. Polybrene® (sc-134220) was prepared to a final concentration of 5 µg/ml. 1 mL of this mixture is used to replace the media in each well. Lentiviral particles were thawed at room temperature and added to culture. The solution was swirled to mix properly, and the plate was incubated overnight. The culture medium was replaced with 1 ml of regular medium. Cells were incubated overnight. Stable clones expressing the shRNA were split 1:3 and incubated

for another 24-48 hours in complete medium. Cells underwent puromycin selection using a concentration ranging from 2 to 10 ug/mL to kill the non-transduced cells. Media was changed with fresh puromycin-containing medium every 2-3 days.

D. **Western Blot Verification of Grx1 and Grx2 Knockdown**

Cells were lysed, and proteins were extracted using RIPA buffer and protease inhibitor. Protein concentration was determined by the BCA Protein Assay. 60 micrograms of control HepG2, control shRNA, and Grx1 shRNA HepG2 was loaded into the lanes of a 15% gel and then blotted onto a PVDF membrane using the wet transfer method. The membrane was then tested for GAPDH as a loading control and Grx1.

Mitochondrial isolation using the Dounce homogenization procedure was performed. Forty micrograms of the mitochondrial fractions of control shRNA HepG2 and Grx2 shRNA HepG2 were loaded onto a 15% gel, blotted onto a PVDF membrane, and then tested for VDAC (loading control) and Grx2 antibodies.

Using ImageLab software, the volume of the bands were determined and corrected using the control HepG2 band as a reference band. Multiple samples were analyzed throughout this study to ensure a knockdown of at least 40%.

E. **Doxorubicin Sensitivity: WST-8 Assay**

Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were passaged using routine procedure. To count the cells, 600 ul of PBS and 30 ul of culture were added into a tube. Tube was rotated up and down and shaked to mix the cells within the tube. 10 ul of the solution was added to the crytometer, and cells were counted in each quadrant. Dilution and cell density was

determined and corrected to obtain about $10^3 – 10^4$ cells/mL. 90 uL of cell culture is pipetted into a 96 well plate. After 24 hour incubation, the following groups were made: the first row was control (media only), the second row was cells + 1 uM doxorubicin, and the third row was cells + 10 uM doxorubicin. There were six replicates of scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 for each treatment group. After a 24 hour incubation with each treatment, 10 uL of WST-8 Assay Reagent to each well. Results were analyzed and appropriate calculations were made to determine cell viability (adjusted OD value/control group OD value * 100%). Images of each respective group was taken using a light microscope for morphology analysis.

F. **Western Blot: Apoptotic Pathway**

Cells were lysed, and proteins were extracted using RIPA buffer. Protein concentration was determined by the BCA Protein Assay. 60 micrograms of wildtype HepG2, scramble shRNA, and Grx1 shRNA HepG2 with and without 10 uM doxorubicin was loaded into the lanes of a 15% gel and then blotted onto a PVDF membrane using the wet transfer method. The membrane was then tested for pro-apoptotic Bax, anti-apoptotic Bcl2, and late apoptotic marker cleaved caspase-3. Using ImageLab software, the volume of the bands were determined and corrected using the control HepG2 band as a reference band.

G. **Western Blot: Nrf2 Pathway and Nrf2's Downstream Genes**

Cells were lysed, and proteins were extracted using RIPA buffer. Protein concentration was determined by the BCA Protein Assay. 60 micrograms of scramble shRNA HepG2, Grx2 shRNA HepG2, and Grx1 shRNA HepG2 with and without 10 uM doxorubicin for 24 hours was loaded into the lanes of a 15% gel and then blotted onto a PVDF membrane using the wet transfer method.

The membrane was then tested for Nrf2 and its downstream genes including heme oxygenase-1 (HO-1), NADPH dehydrogenase quinone-1 (NQO1), catalase, superoxide dismutase, and thioredoxin. Using ImageLab software, the volume of the bands were determined and corrected using the control HepG2 band as a reference band.

H. **Western Blot: Mitochondrial Antioxidant Proteins**

HepG2 scramble shRNA and Grx2 shRNA mitochondria were prepared using Dounce homogenization mitochondrial isolation. 40 micrograms of control shRNA HepG2 and Grx2 shRNA HepG2 was loaded into the lanes of a 15% gel and then blotted onto a PVDF membrane using the wet transfer method. The membrane was then tested for Nrf2 mitochondrial downstream genes including thioredoxin 2 (Trx2) and peroxiredoxin 3 (PRDX3). Using ImageLab software, the volume of the bands were determined and corrected using COX IV.

I. **PSSG Western Blot: PSSG Accumulation and Actin Glutathionylation**

Cells were lysed, and proteins were extracted using RIPA buffer. Protein concentration was determined by the BCA Protein Assay. 60 micrograms of control shRNA HepG2, Grx2 shRNA HepG2, and Grx1 shRNA HepG2 treated with and without 10 uM for 2 hours was loaded into the lanes of a 15% gel and then blotted onto a PVDF membrane using the wet transfer method. The membrane was then tested for PSSG and then reprobed for actin. Using ImageLab software, the volume of the bands were determined and corrected using the control HepG2 band as a reference band.

Similarly, mitochondrial portions of Grx1 shRNA, Grx2 shRNA, and control shRNA treated with and without 10 uM of doxorubicin treatment for 2 hours were isolated using the

Dounce homogenization method and then 60 micrograms were loaded onto a 15% gel and blotted onto a PVDF membrane. The membrane was then incubated with anti-PSSG antibody to see whether glutathionylation increased with Grx1 and Grx2 knockdown for mitochondrial proteins.

J. **Mitochondrial PSSG and Mass Spectrometry: Grx2's Mitochondrial Targets**

Mitochondria from scramble shRNA and Grx2 shRNA with 10 uM doxorubicin treatment for 1.5 h using the Dounce Homogenization method was isolated. The mitochondrial samples were then used to pull down GSH using the anti-GSH antibody (Invitrogen). The samples were run on a 15% gel and stained using Coomassie Brilliant Blue stain. Mitochondria was isolated from scramble shRNA and Grx2 shRNA after 10 uM doxorubicin treatment for 1.5 h, and samples were run on an immunoblot. The immunoblot was probed with anti-PSSG antibody and analyzed. Seven corresponding bands between 100-150 kDa, 50 kDa, and 15-20 kDa were selected and excised from the gel to further process through mass spectrometry for identification.

K. **Nrf2-Grxs and Nrf2-SSG Co-immunoprecipitation**

Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 were treated with and without 10 uM doxorubicin for 1.5 h. Nrf2 was pulled down in all the samples using the Nrf2 antibody (Santa Cruz) using the magnetic beads procedure. Samples were then run on a 15% gel, blotted onto a membrane, and then probed with anti-Nrf2, anti-PSSG, anti-Grx1, and anti-Grx2 antibodies.

L. **Nrf2 and Glutaredoxin Translocation Assay**

Cytoplasmic and nuclear fractions were isolated using the Nuclear Extraction Kit (Thermo Scientific) from scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 with and without

doxorubicin treatment in a time-dependent manner. Two different time points were assessed: 0 and 6 h. Nuclear extracts were analyzed for Nrf2, Grx1, Grx2, VDAC, and lamin A/C using western blot. Cytoplasmic extracts were analyzed for Nrf2, Grx2, and GAPDH using western blot. Bands were analyzed using BioRad's ImageLab software.

M. **Grx2 Co-localization and Nrf2 Binding in the Nucleus**

HepG2 was fixed using 4% paraformaldehyde. HepG2 was incubated with Grx2 antibody (abcam) conjugated to MitoTracker red dye (Life Technologies). DAPI (Life Technologies), a nuclear stain, was used to counter stain. Images were taken with a confocal microscope.

HepG2 was fixed using 4% paraformaldehyde. HepG2 was incubated with Grx2 antibody (abcam) conjugated to Alexa 594 dye, Nrf2 antibody (Santa Cruz) conjugated to Alex 488 antibody, and counterstained with DAPI nuclear stain (Life Technologies). The images were taken with a confocal microscope.

N. **NucBlue Live Cell Stain**

Scramble shRNA HepG2 and Grx2 shRNA HepG2 cells were grown in a 6 well plate to a 90% confluency. One well of scramble shRNA and Grx2 shRNA HepG2 cells were incubated with 5 uM doxorubicin and another well was incubated with 10 uM doxorubicin for 24 h. The third well was kept as a control (no doxorubicin treatment). Two drops of NucBlue live cell stain was added to each well after 24 h doxorubicin incubation and then imaged after 20 minutes using a confocal microscope.

O. **Antioxidant Gene Screening: Antioxidant Enzyme Pathway Panel PrimePCR Assay**

91 Nrf2-pathway related genes including survival genes and Nrf2's downstream targets were analyzed using the Antioxidant Enzyme Pathway Panel PrimePCR Assay from BioRad. Total RNA was isolated from scramble shRNA HepG2, Grx1 shRNA HepG2, and Grx2 shRNA HepG2 with and without 10 uM doxorubicin treatment for 24 h using the Aurum total RNA mini kit. cDNA was synthesized from these six samples using the iScript family of reverse transcriptase reagents. Six plates were run through the qPCR machine and analyzed via the CFX Manager software (BioRad). Data was equalized to the GAPDH mRNA data.

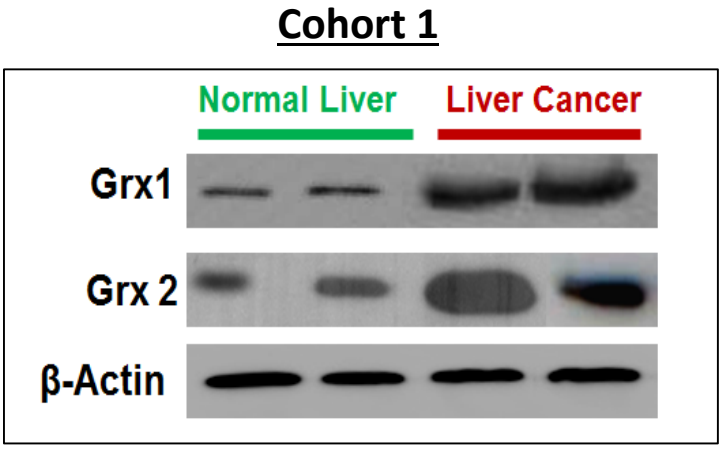
RESULTS

Glutaredoxin is overexpressed in hepatocellular carcinoma, and shRNA-mediated knockdown caused about a 50-70% decrease in glutaredoxin level in HepG2.

The glutaredoxin redox state in cancer especially liver cancer is relatively unknown. Very few research studies published in the last two decades discusses the function and importance of glutaredoxin in liver cancer. Thus, to determine glutaredoxin expression in hepatocellular carcinoma patients, we collaborated with Sun Yat-Sen Hospital in China and collected surgically resected tissue from five patients. Each patient had both cancerous liver tissue and neighboring healthy tissue taken and tested for Grx1 and Grx2 expression level. This method was used to ensure limited variation of the results. As seen in fig. 4, Grx1 and Grx2 are highly overexpressed in hepatocellular carcinoma in both cohorts of the clinical study. Many studies support that glutaredoxin is vital for the survival of the cell and has special anti-apoptotic abilities especially in times of oxidative stress [55]. Moreover, breast and prostate cancer in numerous studies have shown to have increased levels of glutaredoxin as well [45].

Knowing that glutaredoxin may be vital for cancer cell survival, we decided to knockdown glutaredoxin and establish specific HepG2 glutaredoxin knockdown cell lines to later decipher whether glutaredoxin knockdown affects HepG2 cancer survival especially after doxorubicin treatment. We used a lentivirus to carry the scramble shRNA, Grx1 shRNA, and Grx2 shRNA particles into the cell. After puromycin selection, certain colonies and mixes of colonies were selected on random. These cells were lysed, and protein was collected to test for Grx1 and Grx2 protein level. Since Grx2 is largely concentrated in the mitochondria, mitochondrial isolation was performed prior to protein extraction. Because of the transience of shRNA, Grxs protein level tends to fluctuate, as seen in Fig. 5 and Fig. 6. This fluctuation is attributed to two factors: shRNA

instability and the Grxs essential function in the cell. shRNA is a preferred method to siRNA because its small hairpin loop makes it less prone to degradation and hence, longer lasting. However, shRNA is still considered short-acting, and its effects may vary from colony to colony [56]. Because Grxs are vital for cell survival and growth, it would be difficult to also fully knockdown Grxs in the cell. However, most transfections allowed for a 50-70% knockdown as seen in Fig. 5 and Fig. 6. Because of the difficulty in maintaining this percent knockdown, transfections are continuously performed and tested via western blot for expression level. The most successful transfections were used for further experimentation. Overall, glutaredoxin was shown to be overexpressed in liver cancer, and shRNA transfections giving a 50-70% knockdown of Grx1 and Grx2 shRNA were established.



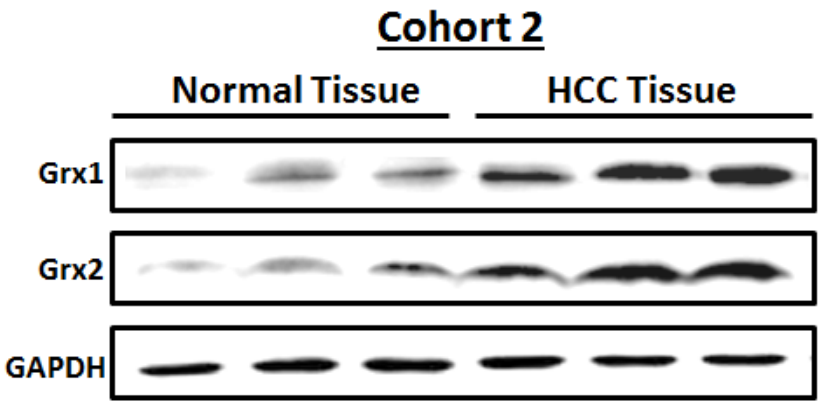


Fig 4. Grx1 and Grx2 are highly expressed in liver cancer. Cancerous liver tissue and neighboring healthy liver tissue was obtained from five patients at Sun-Yat-Sen Hospital. These ten tissue samples were then tested for Grx1 and Grx2 protein level expression using western blot. Grx1 and Grx2 are highly expressed in cancerous tissue compared to normal liver tissue.

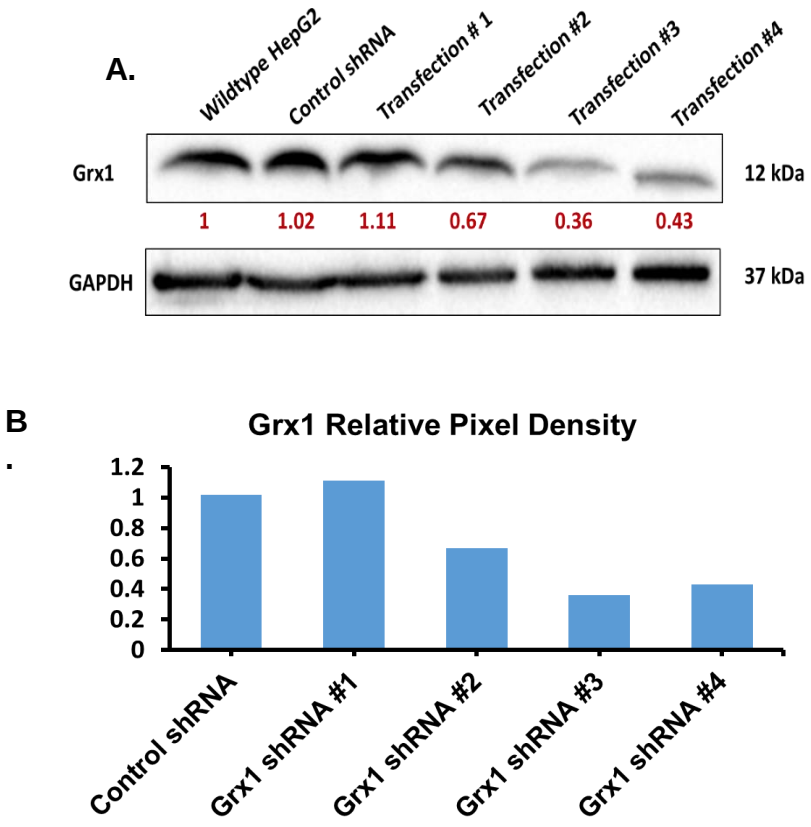


Fig 5. Grx1 knockdown via shRNA gave a 50-60% knockdown. A.) Western Blot analysis of four different transfections of Grx1 shRNA HepG2 cells, scramble shRNA HepG2, and wildtype HepG2 cells was performed. Scramble shRNA and Grx1 shRNA HepG2 cells were transfected using a lentivirus carrier. Following protein extraction, SDS-PAGE and analysis was performed. B.) Quantification of western blot analysis and normalized to the GAPDH control. (n=6)

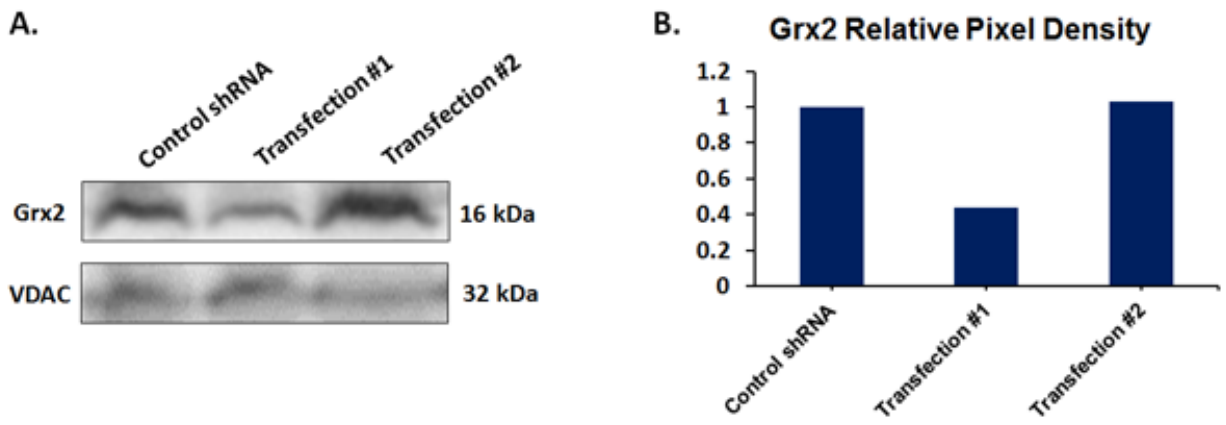


Fig 6. Grx2 knockdown via shRNA gave about a 60% knockdown. A.) Western Blot analysis of two different transfections of Grx2 shRNA HepG2 cells and scramble shRNA (control shRNA) was performed. Scramble shRNA and Grx2 shRNA HepG2 cells were transfected using a lentivirus carrier. Following mitochondrial isolation and protein extraction, SDS-PAGE and analysis was performed. B.) Quantification of western blot analysis and normalized to the VDAC control. (n=6)

Glutaredoxin inhibition sensitizes HepG2 to doxorubicin

Using our Grx1 and Grx2 shRNA HepG2 cell lines, we wanted to validate the first part of our hypothesis that Grx1 and Grx2 knockdown would sensitize HepG2 to doxorubicin. The first method chosen for our study was the WST-8 assay. The WST-8 assay is a colorimetric cell viability assay. Six replicates of 1 uM and 10 uM doxorubicin-treated scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 were used to increase the authenticity of our results. As shown in fig. 7, Grx1 and Grx2 shRNA HepG2 cells have significantly lower cell viabilities than scramble shRNA. At 1 uM doxorubicin, scramble shRNA tends to have 90% viable cells, but Grx1 shRNA and Grx2 shRNA HepG2 groups have a cell viability of 70-80%. At 10 uM doxorubicin, scramble shRNA have about a 60% cell viability while Grx1 and Grx2 shRNA have about a 40-50% cell viability. Morphological data, as seen in fig. 8, shows similar results. Cell numbers are severely decreased after 10 uM doxorubicin treatment in Grx1 and Grx2 shRNA HepG2 cell lines.

However, scramble shRNA cells with doxorubicin treatment have relatively unchanged cell numbers compared to without treatment. Nonetheless, it is evident that Grx1 and Grx2 shRNA HepG2 are more sensitive to doxorubicin treatment than control HepG2 cells.

This study was expanded to include crucial apoptotic proteins. Bcl2 is an anti-apoptotic factor that inhibits Bax, a pro-apoptotic protein, early in the apoptotic pathway. Cleaved caspase-3 is often used as a late apoptotic marker. Cleaved caspase-3 is the point at which apoptosis is the guaranteed pathway for the cell. As seen in fig. 9, Bcl2 is highly expressed in HepG2 cells, but with Grx1 and Grx2 knockdown, Bcl2 is slightly decreased yet remains relatively high. Upon 10 uM doxorubicin treatment, Bcl2 for scramble shRNA HepG2 cells decreases compared to without treatment but is significantly higher than Grx1 and Grx2 shRNA HepG2 cell lines. In fact, densitometry shows that scramble shRNA with doxorubicin treatment has relatively the same Bcl2 expression level as Grx1 and Grx2 shRNA HepG2 cell lines. A high Bcl2 level is a common method used in multiple cancer cell lines to thwart the effectiveness of anti-cancer drugs because of Bcl2's ability to protect the cell, and Grx knockdown helps decrease Bcl2 level [57]. Bax showed the opposite pattern of results. Bax is relatively low in all untreated cells, but with doxorubicin treatment, Grx1 and Grx2-inhibited cells are the most affected with a significant increase in Bax levels compared to control. Scramble shRNA with doxorubicin treatment has a low Bax level that is very similar to untreated groups. The relatively unchanged Bax and Bcl2 levels in scramble shRNA HepG2 with and without doxorubicin treatment shows the high anti-apoptotic resistance in HepG2 cells, but with Grxs inhibition, this resistance is relatively reversed especially after doxorubicin treatment. Cleaved caspase-3 shows similar results to Bax. Cleaved caspase-3 is nonexistent in untreated groups but is highly expressed in Grx1 and Grx2 shRNA

HepG2 cell lines after doxorubicin treatment compared to scramble shRNA. All these results tend to show that Grx1 and Grx2 inhibition allows for higher sensitivity to doxorubicin.

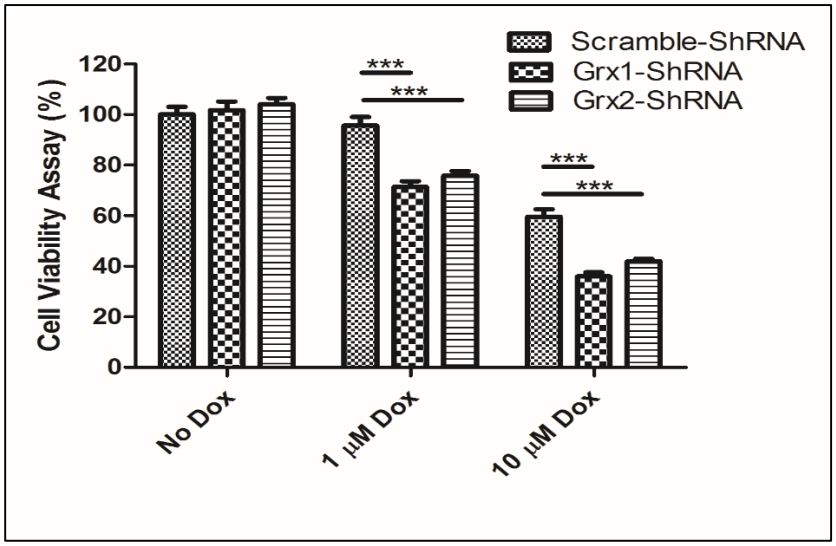


Fig 7. Grxs inhibition causes increased sensitivity to 1 uM and 10 uM doxorubicin than normal HepG2 cells. Grx1 shRNA, Grx2 shRNA, and scramble shRNA HepG2 cells were seeded to $1x10^6$ cells/well on a 96-well plate for 24 hours, and then six wells of each group was treated with either no doxorubicin, 1 uM doxorubicin, and 10 uM doxorubicin for 24 hours. Cells were incubated with WST-8 reagent for 2 hours, and OD_{450} was measured via spectrometer. Quantification of WST-8 assay results are shown as mean $\pm$ SEM (n = 6). Statistical analysis was performed using unpaired Student's t-test. (ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

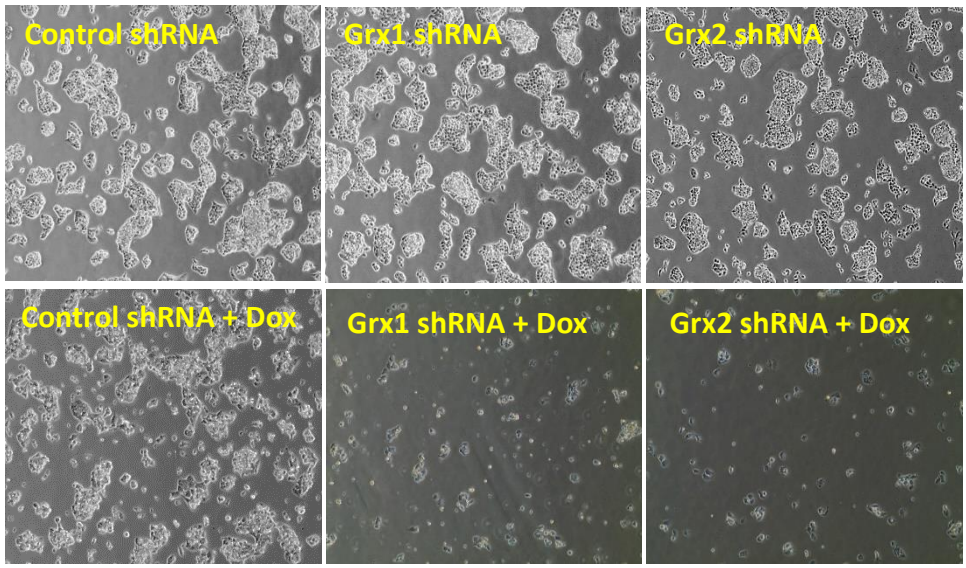


Fig 8. Grxs inhibition causes decreased cell numbers after 10 uM doxorubicin treatment than in control HepG2 cells. Morphological analysis of control shRNA (scramble shRNA), Grx1 shRNA, and Grx2 shRNA HepG2 cells with and without 10 uM doxorubicin treatment for 24 hours. Control shRNA with doxorubicin treatment have higher cell numbers than Grx1 and Grx2 shRNA HepG2 cells after doxorubicin treatment.

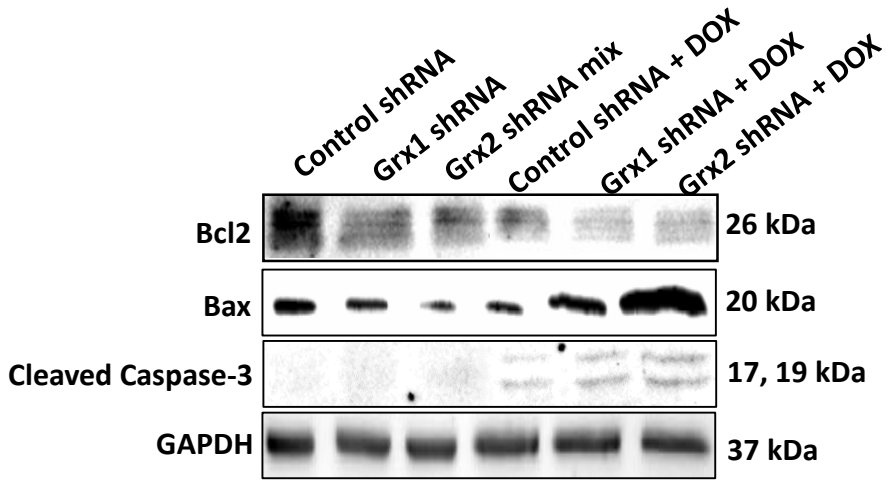


Fig 9. Grxs inhibition caused increased Bax and cleaved caspase-3 levels and lower Bcl2 levels after doxorubicin treatment. Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin. Upon treatment, the cells were incubated for 24 hours. Following incubation, proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for Bcl2, Bax, cleaved caspase-3, and GAPDH antibodies. b) Quantification of western blot analysis using densitometry, and normalized to the GAPDH control. (n=3)

PSSG Accumulation in Grx1 and Grx2 shRNA HepG2 cells

Protein glutathionylation (PSSG) is abundant during times of oxidative stress and may critically alter cellular function, particularly for cell death [12]. PSSG is relatively fast-acting, so time and doxorubicin concentration precision was quite important. Multiple concentrations and incubation times were used to optimize PSSG detection. Scramble, Grx1, and Grx2 shRNA HepG2 cells were incubated with and without 10 uM doxorubicin for 2 hours. This incubation time and concentration showed the most ideal PSSG accumulation. Before treatment, scramble shRNA, Grx1, and Grx2 shRNA have relatively low PSSG accumulation, but after doxorubicin treatment,

Grx1 shRNA have a distinct red smear from 25 kDa to 10 kDa, as seen in fig. 10. There is also distinct PSSG accumulation in the heavier proteins as well. Grx2 shRNA HepG2 after doxorubicin treatment also has a small PSSG smear but not as prominent as Grx1 shRNA HepG2's smear. Scramble shRNA after doxorubicin treatment has relatively little PSSG accumulation. There were a couple of bands of interest, but in particular, there was one band located in between 37 and 50 kDa. Because actin has a molecular weight of 42 kDa, we decided to reprobe the membrane for actin. After reprobing for actin, we did a merged picture to see the overlay between PSSG and actin. Highly glutathionylated actin has a perfect merge, which gives a yellow color. As seen in fig. 10, actin glutathionylation increases from left to right. This distinct yellow actin band indicates that Grx1 and Grx2 shRNA may stimulate actin glutathionylation.

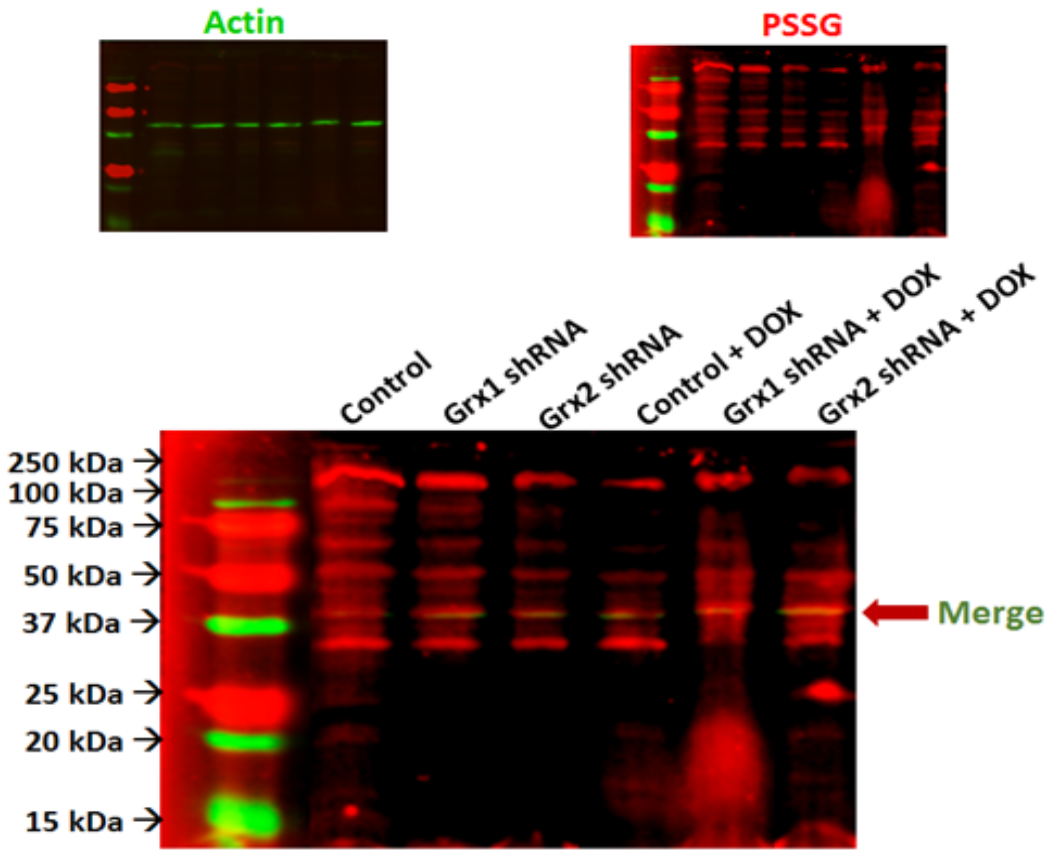


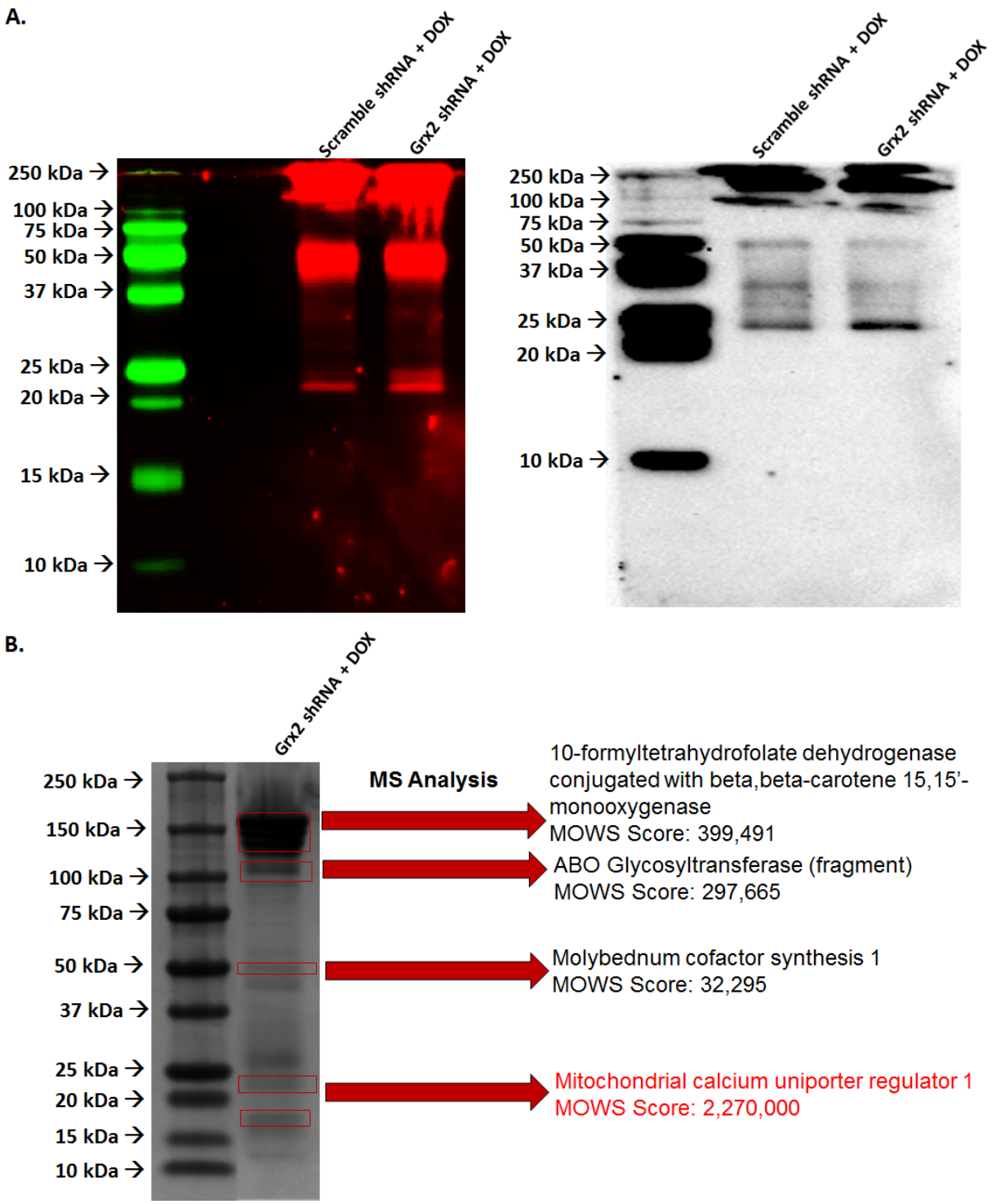
Fig 10. Grxs inhibition increases PSSG accumulation and actin glutathionylation after doxorubicin treatment. Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin. Upon treatment, the cells were incubated for 2 hours. Following incubation, proteins were extracted, ran on a 15% gel, and blotted onto a membrane. The membrane was then tested for anti-GSH antibody and reprobed with anti-actin antibody. Western blot analysis was then performed.

Identification of Grx2's mitochondrial targets using mass spectrometry

Grx2 is highly concentrated in the mitochondria, amounting to be at least 10 times more concentrated there than any other organelle [18]. Currently, it is well know that Grx2 protects and repairs complex I and IV, but considering that the mitochondria is heavily involved in apoptotic pathway, Grx2 may also have other protein targets of interest that may regulate apoptosis and protect the cell [23, 24]. As such, western blot and mass spectrometry was performed to identify potential Grx2 targets. Both fluorescence and HRP imaging was done to ensure that all possible glutathionylated mitochondrial proteins would appear (Fig. 11A). There was slightly more glutathionylation in Grx2 shRNA with doxorubicin treatment especially a ~25 kDa band that was slightly more glutathionylated in Grx2 shRNA (Fig. 11A).

Moreover, glutathione immunoprecipitation on mitochondrial samples were conducted and ran on a gel that was then incubated with the Coomassie Blue stain to illuminate the glutathionylated protein bands (Fig. 11B). Seven bands of interest were then chosen and excised to identify through mass spectrometry. Three bands were taken from greater than 100-175 kDa and were speculated to be 10-formyltetrahydrofolate dehydrogenase conjugated with beta, beta-carotene-15,15'-monooxygenase, which corresponded nicely to the band position and protein weights. It also had a MOWS score of 399,491 (Fig. 11B). These enzymes are somewhat correlated in cancer as possible pharmacological targets. 10-formyltetrahydrofolate dehydrogenase (10-FTHD) is normally considered to be downregulated in hepatocellular carcinoma as well as multiple

other cancer cell lines compared to their noncancerous counterpart [58]. 10-FTHD is an enzyme involved in folate metabolism, often decreasing the amount of 10-FTH in the cell. 10-FTH is an important precursor for de novo purine biosynthesis and cellular proliferation, both important for cancer growth and survival. Inhibition of 10-FTHD increases the amount of available 10-FTH in the cancer cell [58]. Beta-beta-carotene-15,15'-monoxygenase is an enzyme involved in the conversion of beta-carotene to retinaldehyde which can later form retinoic acid. Retinoic acid is vital to stop cancerous growth because it can inhibit matrix metalloproteinases [59]. The fourth band was determined to be most likely ABO glycosyltransferase (Fig. 11B). Because this is an enzyme found only in red blood cells and the weight does not correspond with the position of the band, this was said to be a nonsensical match [60]. The fifth band excised was at approximately 50 kDa and was said to be Molybdenum Cofactor Synthesis 1 with a MOWS score of 32,295 (Fig. 11B). Because of this low MOWS score, this was considered to be an unreliable match. The sixth and seventh bands between 15-25 kDa correspondingly identified mitochondrial calcium uniporter regulator 1 (MCUR1) (Fig. 11B). MCUR1 is a 40 kDa molecule with two transmembrane domains in the mitochondrial membrane and a coiled coil domain in the mitochondrial matrix [61]. Because two bands had the highest MOWS score (2,270,000) for this protein, it is possible that degradation occurred, causing two bands to result (Fig. 11B). Also, the band near 50 kDa named mitochondrial uniporter regulator 1 as a second likely match. The mitochondrial uniporter regulator 1 is unique in that it regulates mitochondrial uniporter 1, an enzyme involved in the uptake of calcium into the mitochondria [61, 62]. Previous studies have shown that inhibition of MCUR1 results in decreased oxidative phosphorylation, low cellular ATP levels, and autophagy [62]. Immunoprecipitation and western blots will have to be conducted to confirm that these proteins are truly glutathionylated and also Grx2 targets.


A.
Scramble shRNA + DOX
Grx2 shRNA + DOX
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Scramble shRNA + DOX
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Grx2 shRNA + DOX
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20 kDa
15 kDa
10 kDa
MS Analysis
10-formyltetrahydrofolate dehydrogenase conjugated with beta,beta-carotene 15,15'-monooxygenase
MOWS Score: 399,491
ABO Glycosyltransferase (fragment)
MOWS Score: 297,665
Molybednum cofactor synthesis 1
MOWS Score: 32,295
Mitochondrial calcium uniporter regulator 1
MOWS Score: 2,270,000

C.

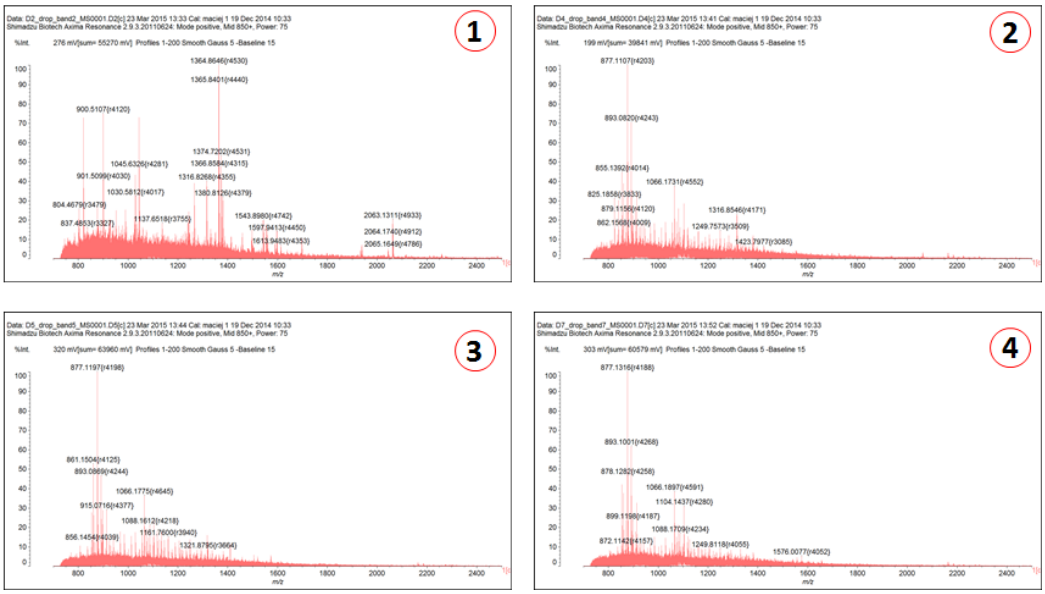

Fig 11. Mass spectrometry identified Grx2's key mitochondrial targets. A.) Mitochondrial extracts of scramble shRNA and Grx2 shRNA with 10 uM doxorubicin treatment for 1.5 h were run on a 15% gel and blotted onto a membrane. The membrane was then tested for anti-GSH antibody using both fluorescent and HRP secondary antibodies. Western blot analysis was performed. B.) Glutathione was pulled down using anti-GSH antibody from mitochondrial extracts of scramble shRNA and Grx2 shRNA with 10 uM doxorubicin treatment for 1.5 h. The samples were run on a pre-made gradient gel and stained with Coomassie Blue stain. The gel was then imaged using BioRad ImageLab software. Seven bands were excised and processed via mass spectrometry. Band likelihood was predicted using the MS/MS peaks and the online Mascot software from Matrix Science. C.) MS/MS peaks formulated and imaged using software of the four select bands from B.

Grxs inhibition decreases Nrf2 and its downstream genes

The second part of my hypothesis states that the reason that glutaredoxin inhibition sensitizes hepatocellular carcinoma to doxorubicin is due to impairment of the Nrf2-dependent antioxidant pathway. To substantiate this claim, we decided to determine Nrf2 expression level in scramble, Grx1, and Grx2 shRNA with and without 10 uM doxorubicin using western blot. Nrf2 in scramble shRNA HepG2 is highly expressed. Previous studies have shown that Nrf2 is highly expressed in cancer cells because Nrf2 needs to be readily available for the transcription of multiple antioxidant enzymes to protect the cancer cell from oxidative damage and increase cell

survival [37]. Nrf2 slightly decreases about 20-30% in Grx1 and Grx2 shRNA without doxorubicin treatment. However, after doxorubicin treatment, control shRNA remains comparatively high, with a Nrf2 level of 62%. Grx1 shRNA HepG2 after doxorubicin treatment has a major decrease in Nrf2 expression level, with a 44% protein expression level (Fig. 12). Grx2 shRNA HepG2 shows a similar decrease, with a 32% Nrf2 expression level. Because there was a noteworthy decrease in Nrf2, we decided to test for Nrf2's downstream genes to see if an analogous decrease would occur. The tested Nrf2's downstream antioxidant enzymes were HO-1 (heme oxygenase-1), NQO1 (NADPH quinone oxidase 1), catalase, and thioredoxin 1 (Trx1). HO-1 is highly expressed in the untreated groups, but after 10 uM doxorubicin treatment, Grx1 and Grx2 shRNA HepG2 have a lower HO-1 level compared to control shRNA, with a HO-1 expression level of about 40-50%. On the other hand, HO-1 level remains high in scramble shRNA with doxorubicin treatment with a 75% expression level (Fig. 13). NQO1 showed similar results. Upon doxorubicin treatment, Grx1 shRNA HepG2 group had a 57% NQO1 expression level while Grx2 shRNA HepG2 group had a 27% NQO1 level (Fig. 14). Scramble shRNA HepG2 still had a good NQO1 expression level, with a 76% expression. Similarly, catalase has high levels in scramble shRNA HepG2 without doxorubicin treatment. Grx1 and Grx2 shRNA HepG2 cells have slightly decreased catalase levels, 67% and 59% respectively. Scramble shRNA after doxorubicin treatment remains high with about a 50% catalase level. After doxorubicin treatment, Grx1 and Grx2 shRNA HepG2 have low levels of catalase, with about a 20-30% expression (Fig. 15). Trx1 (thioredoxin 1) has fluctuating expression levels but relatively shows a similar pattern. In scramble shRNA, Trx1 has a low expression level, but upon doxorubicin treatment (oxidative stress), Trx1 is upregulated nearly 2.5 times as much. Grx1 and Grx2 shRNA HepG2 cell lines have increased Trx1 levels, about 1.5 times as much as scramble shRNA. After doxorubicin treatment, Grx1

shRNA has a Trx1 level that remains rather the same, from 1.37 to 1.88. Grx2 shRNA with doxorubicin treatment decreases considerably to about 98%, which is similar to scramble shRNA without doxorubicin treatment (Fig. 16). Grxs inhibition substantially decreases Nrf2 and its downstream genes like HO-1, NQO1, Trx1, and catalase especially after doxorubicin treatment.

Nrf2 also has downstream targets located in the mitochondria. Grx2 is highly concentrated in the mitochondria and is well known to be a protector of mitochondrial enzymes [23, 32]. To see whether Grx2 knockdown would affect mitochondrial antioxidant proteins, a mitochondrial isolation was performed and proteins were loaded onto a gel, blotted onto a membrane, and tested for Trx2 and peroxiredoxin 3 (Prdx3). Trx2 showed a similar pattern to Trx1, as Trx2 upregulated nearly 4.56 times in Grx2-inhibited cells but decreased to 96% after doxorubicin treatment (Fig. 17). Prdx3 is regulated both by thioredoxin and glutaredoxin and is closely linked to Trx2 detoxification pathway in the mitochondria [34]. Thus, it is upregulated in Grx-inhibited cells by 2 times as much but Prdx3 returns back to baseline levels after doxorubicin treatment (Fig. 16).

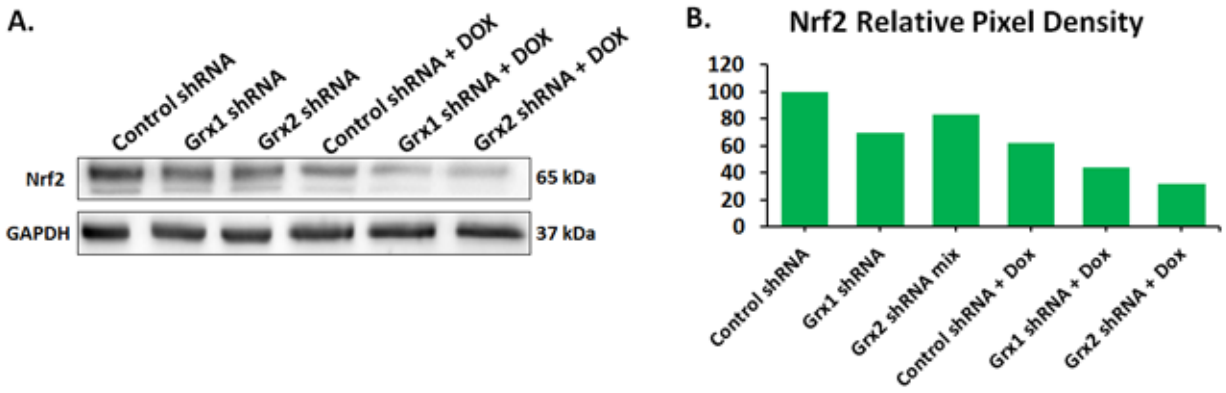

Fig 12. Grxs inhibition decreases Nrf2 expression especially after doxorubicin treatment. A.) Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin. Upon treatment, the cells were incubated for 24 hours. Following incubation, proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for Nrf2 and GAPDH. B.) Quantification of western blot analysis using densitometry, and normalized to the GAPDH control (n = 3).

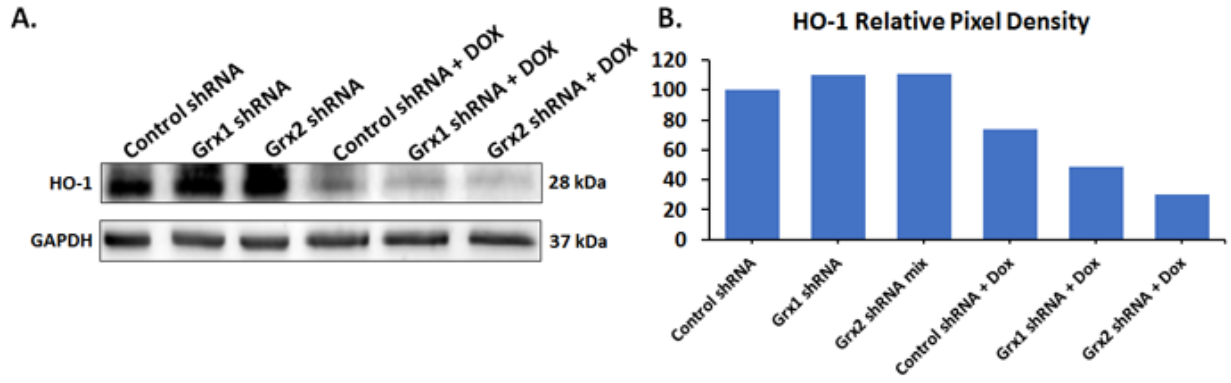

Fig 13. Grxs inhibition decreases Nrf2's downstream gene, HO-1, especially after doxorubicin treatment. A.) Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin. Upon treatment, the cells were incubated for 24 hours. Following incubation, proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for HO-1 and GAPDH. B.) Quantification of western blot analysis using densitometry and normalized to the GAPDH control (n = 3).

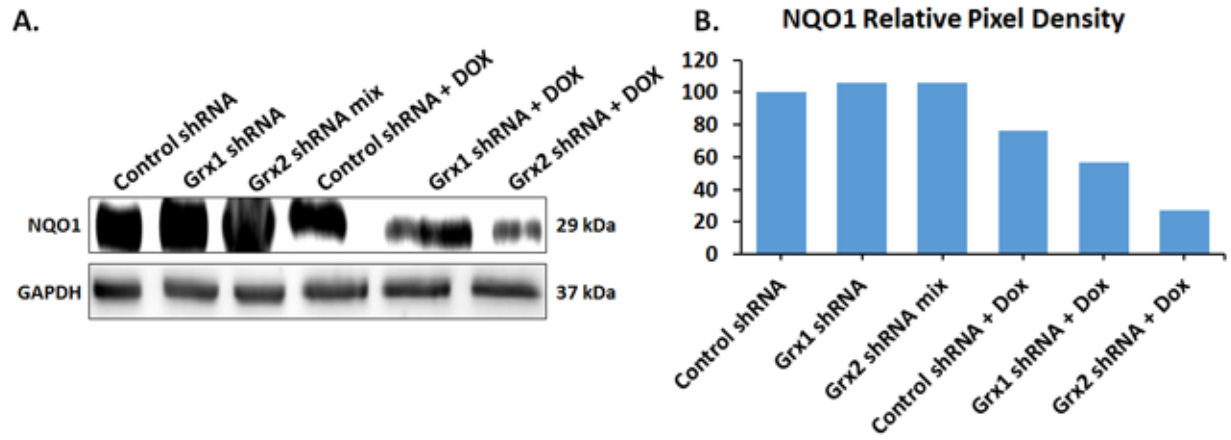

Fig 14. Grxs inhibition decreases Nrf2's downstream gene, NQO1, especially after doxorubicin treatment. A.) Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin. Upon treatment, the cells were incubated for 24 hours. Following incubation, proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for NQO1 and GAPDH. B.) Quantification of western blot analysis using densitometry and normalized to the GAPDH control (n = 3).

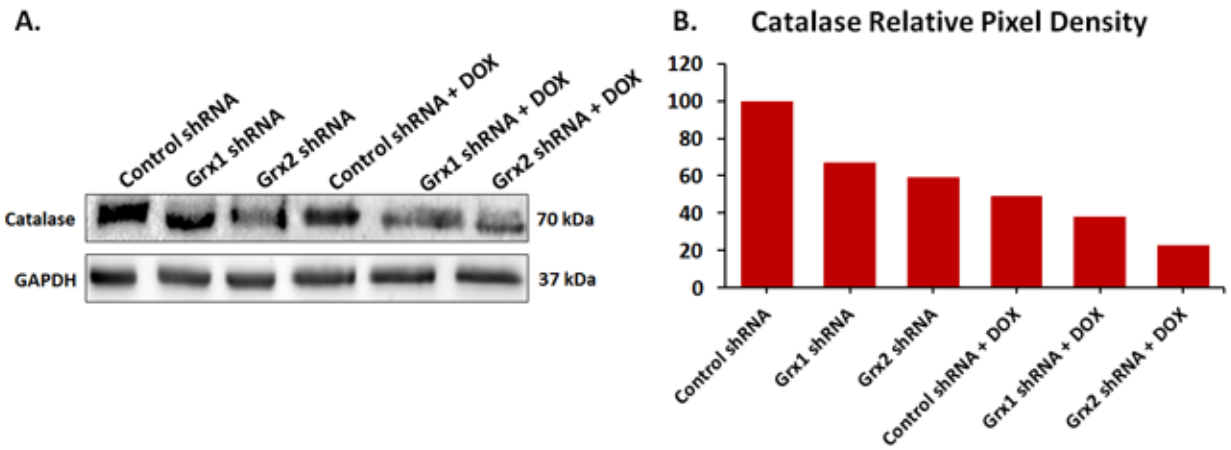

Fig 15. Grxs inhibition decreases catalase expression especially after doxorubicin treatment. A.) Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin. Upon treatment, the cells were incubated for 24 hours. Following incubation, proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for catalase and GAPDH. B.) Quantification of western blot analysis using densitometry and normalized to the GAPDH control (n = 3).

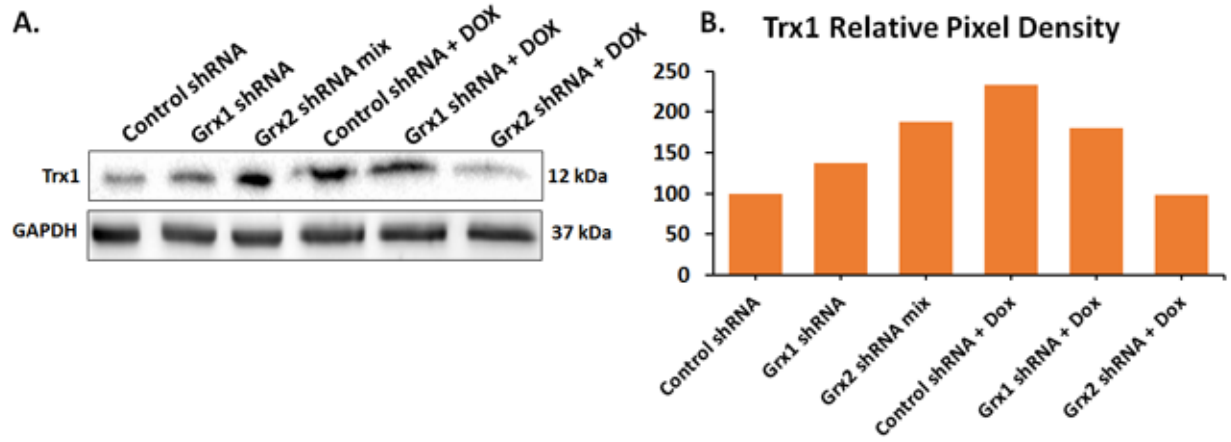

Fig 16. Grxs inhibition increases Trx1 expression without doxorubicin but decreases Trx1 expression after doxorubicin treatment. A.) Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin. Upon treatment, the cells were incubated for 24 hours. Following incubation, proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for Trx1 and GAPDH. B.) Quantification of western blot analysis using densitometry and normalized to the GAPDH control (n = 3).

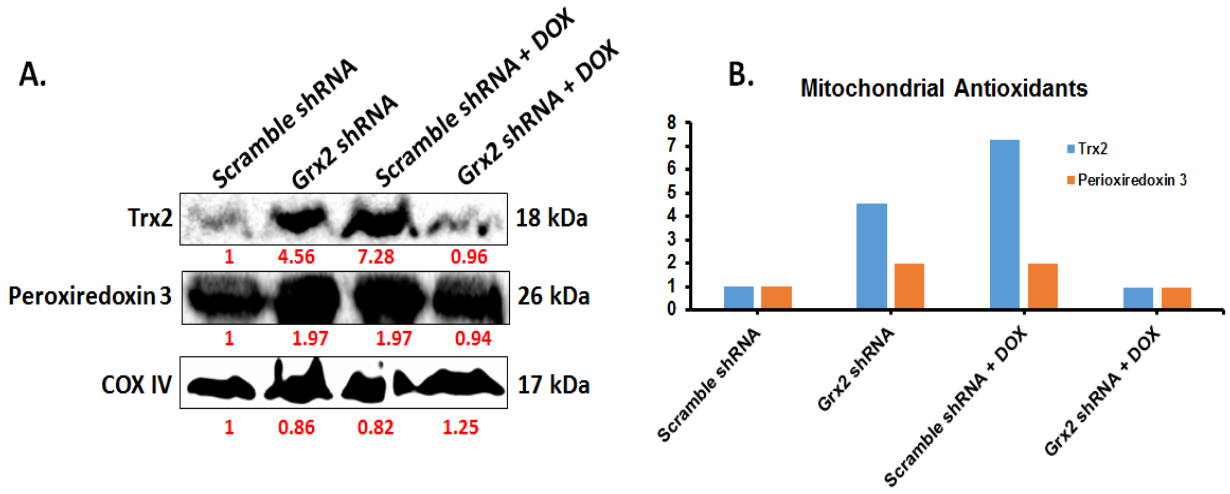

Fig 17. Grxs inhibition increases Trx2 and peroxiredoxin 3 expression without doxorubicin treatment but decreases Trx2 and peroxiredoxin 3 expression after doxorubicin treatment. A.) Scramble shRNA and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin for 24 h. After incubation, mitochondria was isolated via the Dounce homogenization method. Proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for Trx2, peroxiredoxin 3, and COX IV. B.) Quantification of western blot analysis using densitometry and normalized to the COX IV (n = 3).

Doxorubicin increases Nrf2-glutaredoxin binding.

The second part of our hypothesis assumes that Nrf2 is a substrate for glutaredoxin and that in times of oxidative stress, Nrf2-Grx interaction increases to activate Nrf2 (Fig. 3). To validate that Nrf2 may be a substrate for glutaredoxin, a co-immunoprecipitation was performed to pull down Nrf2 and tested on western blot for Grx1 and Grx2 binding. As seen in fig. 18, Nrf2-glutaredoxin binding is very minimal in non-stressed conditions. However, upon doxorubicin stimulation of oxidative stress, Nrf2-Grx1 binding is increased 2.16 times as much, and Nrf2-Grx2 binding is increased 2.34 times as much (Fig. 18).

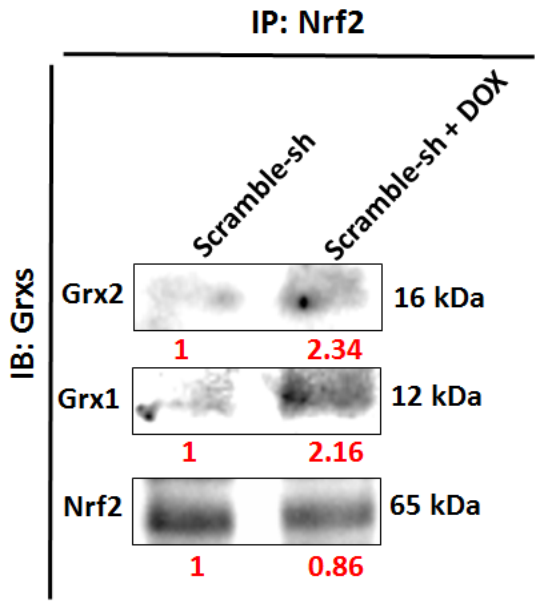

Fig 18. Nrf2 is a substrate for glutaredoxin. Scramble shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin for 6 h. Each sample was used to pull down Nrf2 and then tested on an immunoblot for Grx1, Grx2, and Nrf2. All bands were equalized to the Nrf2 control (n = 3).

Glutaredoxin inhibition increases Nrf2 glutathionylation especially with doxorubicin treatment.

The second part of our hypothesis also relates that Nrf2 is capable of S-glutathionylation and that Grx1 and Grx2 repair Nrf2's glutathionylation so that it can activate ARE. To determine this, an immunoprecipitation pulling down Nrf2 was conducted, and the sample was tested for PSSG on western blot. An incubation period of 15 minutes and a dose of 50 uM doxorubicin was used because doxorubicin distributes within 5 minutes in the cell, and Nrf2 is targeted for ubiquitination every 20 minutes if not activated [39]. As seen in Fig. 19, Nrf2 is highly glutathionylated in scramble shRNA, but upon doxorubicin treatment, Nrf2 glutathionylation decreases. In Grx1 inhibited and Grx2 inhibited HepG2 cells, glutathionylation increases or remains the same with doxorubicin treatment, respectively (Fig. 19). There is also a similar increase in Grx2-inhibited cells after doxorubicin treatment (Fig. 19). Incubation time to obtain

efficient Nrf2 glutathionylation was increased to an hour because Grx2 may work on glutathionylated Nrf2 in the nucleus not repaired by Grx1 or Nrf2 glutathionylation may act as redox sensor to increase trafficking into the nucleus.

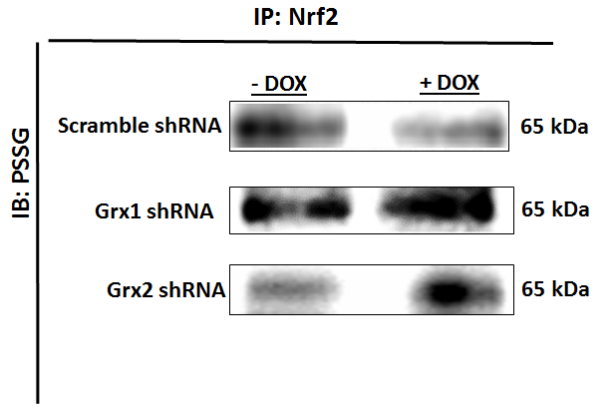

Fig 19. Glutaredoxin inhibition increases PSSG accumulation especially after doxorubicin treatment. Scramble shRNA, Grx1 shRNA, and Grx2 shRNA were treated with 50 uM doxorubicin for 15 minutes. Each sample was used to pull down Nrf2 and then tested on an immunoblot for PSSG.

Grxs inhibition decreases Nrf2's cytoplasmic and nuclear presence

Our previous results indicated that Nrf2 decreases in whole cell lysate (Fig. 12). Nrf2 if not activated can either be degraded in the cytoplasm by the proteasome or can translocate to the nucleus but be unable to bind to ARE due to oxidized cysteine residues [36]. Some studies also support that glutaredoxin is highly expressed in cancer cells that glutaredoxin may be abundant in the nucleus as well [17, 19]. If so, glutaredoxin may be essential in activating Nrf2's cysteine residues in the nucleus to allow for continuous transcription of ARE.

To better elucidate the fate of Nrf2 and the presence of glutaredoxin in the nucleus in glutaredoxin-inhibited HepG2 cells, a nuclear and cytoplasmic extraction was done to test for Nrf2 and glutaredoxin in Grx1 shRNA, Grx2 shRNA, and scramble shRNA HepG2 cells with and

without 10 uM doxorubicin treatment for 6 hours. Nrf2 is highly expressed in the cytoplasm in the untreated groups, but with doxorubicin treatment, Nrf2 is significantly decreased in Grx1 and Grx2 shRNA HepG2 cells, inducing about a 55% Nrf2 expression level (Fig. 19). In the nucleus, a similar pattern is shown, where glutaredoxin-inhibited cells have relatively low Nrf2 expression (Fig. 20) compared to untreated HepG2 groups and doxorubicin-treated scramble shRNA HepG2.

Both glutaredoxin 1 and 2 have shown to be present in the nucleus. Glutaredoxin 1 is present at low levels in both Grx1 and Grx2 shRNA groups with and without doxorubicin treatment, but there is relatively no change in expression level with and without doxorubicin treatment (Fig. 20). Glutaredoxin 2 on the other hand has a distinct pattern in the cytosol and the nucleus. Glutaredoxin 2 has baseline levels in untreated groups, but upon doxorubicin treatment, scramble shRNA HepG2 groups have about a 1.25 times more Grx2 concentration in the nucleus (Fig. 21). However, Grx2 shRNA HepG2 group has nonexistent Grx2 nuclear expression after doxorubicin treatment (Fig. 21). The presence of Grx2 in the nucleus was further verified by testing for VDAC, a mitochondrial marker, and testing for Grx2 in the cytoplasm. There was no VDAC present (Fig. 21) in the nuclear fraction and no Grx2 in the cytoplasm (Fig. 20), supporting that Grx2 may be present in the nucleus.

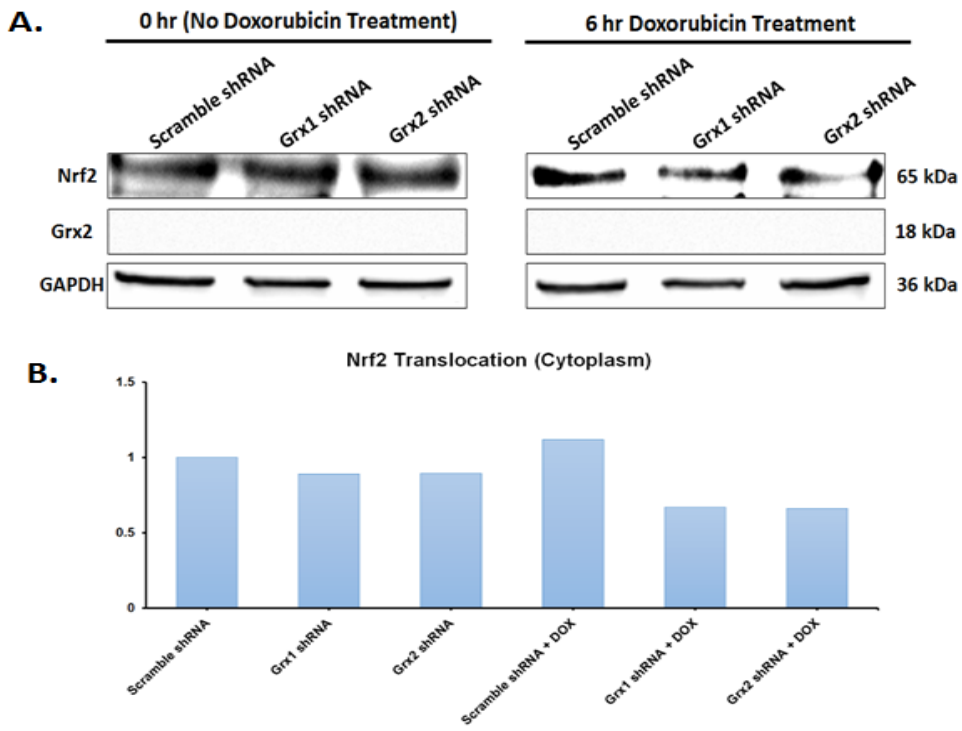

Fig 20. Glutaredoxin inhibition increases Nrf2's degradation in the cytoplasm after doxorubicin treatment. A.) Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin for 6 h. After incubation, the cytoplasmic fraction was extracted using the cytoplasmic and nuclear extraction kit. Cytoplasmic proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for Nrf2, Grx2, and GAPDH. B.) Quantification of Nrf2's bands using densitometry and normalized to the GAPDH (n = 3).

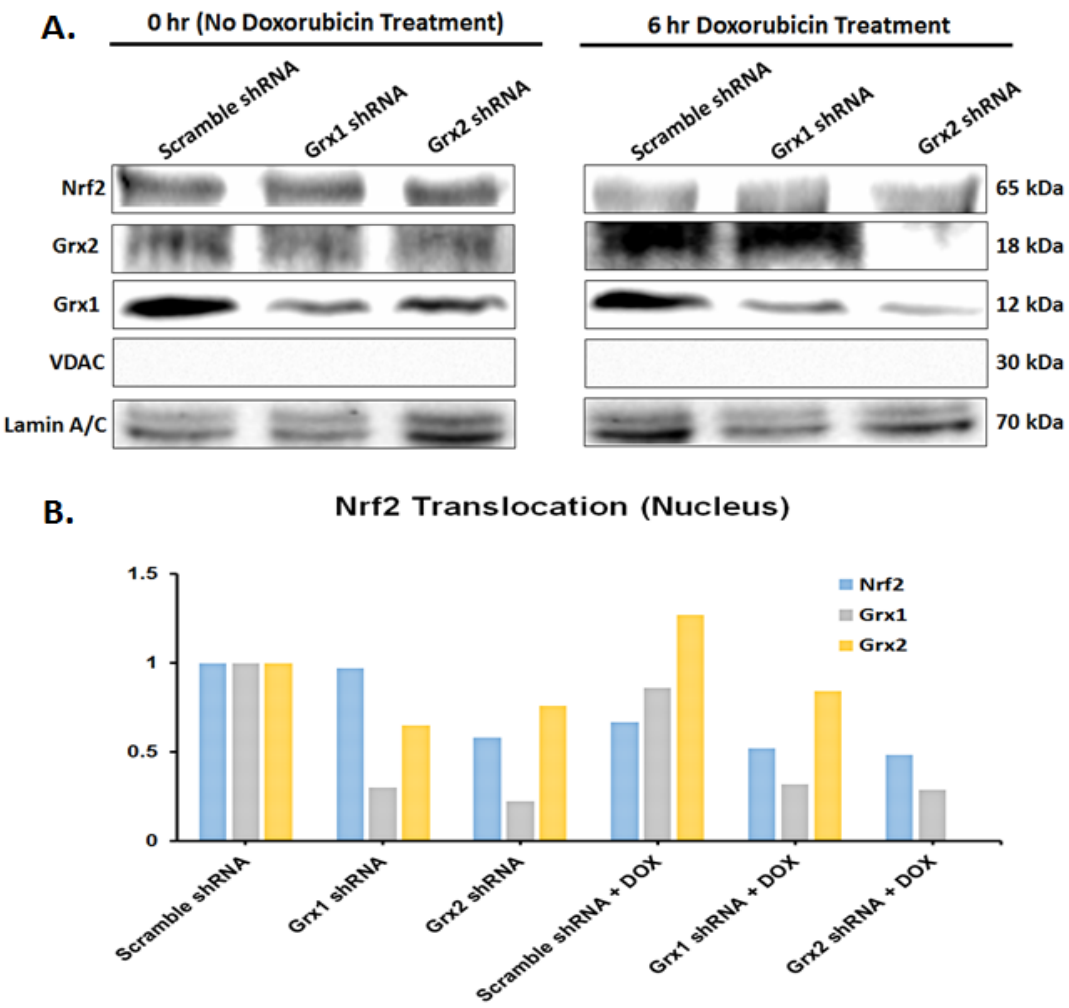

Fig 21. Glutaredoxin inhibition decreases Nrf2's and glutaredoxin's presence in the nucleus after doxorubicin treatment. A.) Scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 cells were plated and incubated overnight at 37°C and 5% CO2. After incubation, one of two treatments was applied: no treatment (negative control) or 10 uM doxorubicin for 6 h. After incubation, the nuclear fraction was extracted using the cytoplasmic and nuclear extraction kit. Nuclear proteins were extracted, ran on SDS-PAGE, and blotted onto a membrane. The membrane was then tested for Nrf2, Grx1, Grx2, VDAC, and lamin A/C. B.) Quantification of western blot analysis using densitometry and normalized to the lamin A/C (n = 3).

Grx2 co-localizes and binds to Nrf2 in the nucleus.

Although Grx2a is ubiquitously located in the nucleus, other studies support that Grx2b and Grx2c may be highly expressed in the nucleus of certain cancer cells [19]. Moreover, if Grx2 was located in the nucleus, it could explain the effect it has on Nrf2's activation of the ARE. Thus,

an immunostaining procedure was done using HepG2 and viewed under the confocal microscope. As seen in Fig. 22, DAPI blue and MitoTracker Red overlap to form a specific purple hue in the nucleus. This strongly indicates that Grx2 is present in the nucleus and in abundant amounts.

To determine whether Grx2 and Nrf2 bind in the nucleus, a triple immunostaining procedure was performed (Fig. 23). Grx2 was tagged using a red fluorescent Alexa 594 antibody, and Nrf2 was tagged using a green fluorescent Alexa 488-conjugated antibody. DAPI was used as a counterstain, which gave a blue fluorescent color. When Nrf2 and Grx2 immunostaining was merged, a yellow color dominated particularly in the nucleus, but red particles of color in the cytoplasm indicated Grx2 in the mitochondria (Fig. 23). When all three stains were merged, a significant white color appeared, indicating that both Nrf2 and Grx2 were present in the nucleus (Fig. 23). This data highly suggests that Grx2 may be essential in repairing Nrf2 glutathionylation in the nucleus.

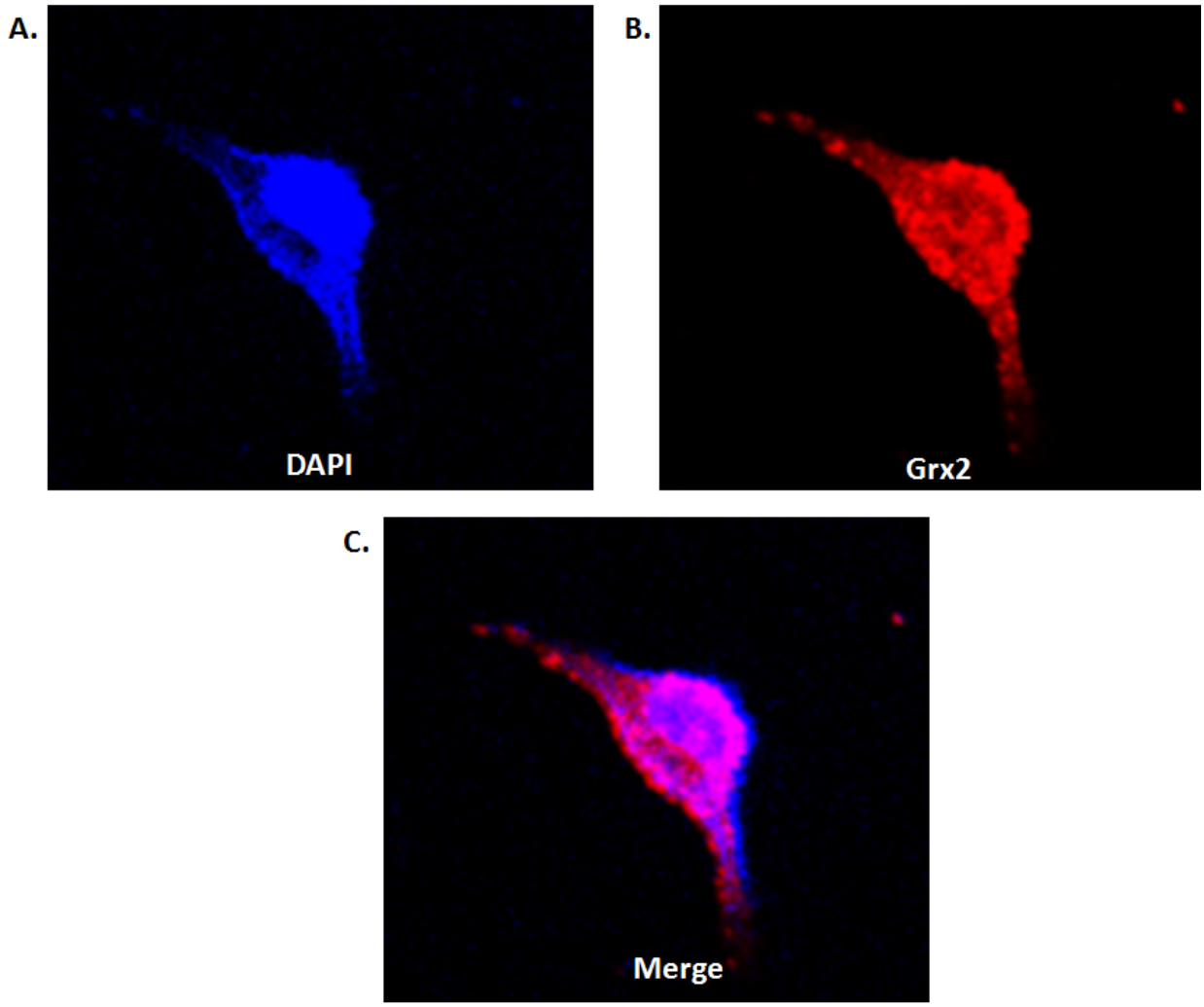

Fig 22. Grx2 is present in the nucleus. (A) HepG2 cells were stained with DAPI to view the nuclei (blue). (B) HepG2 cells were incubated with Grx2 antibody conjugated with MitoTracker Red dye. (C) Merged overlay of DAPI and MitoTracker Red stains produced a purple hue in the nucleus, supporting that Grx2 is present in the nucleus.

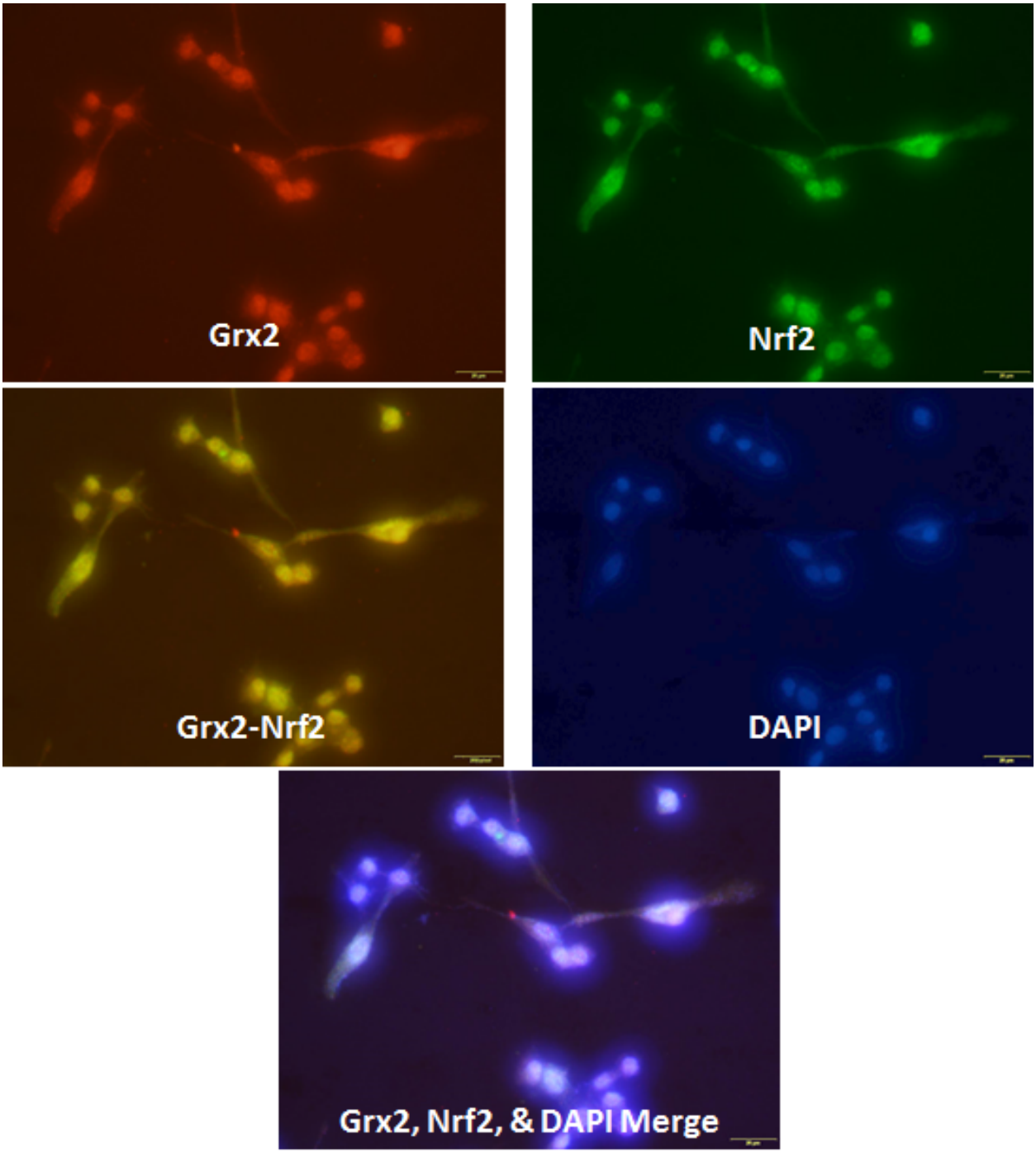

Fig 23. Grx2 repairs Nrf2 in the nucleus. HepG2 cells were incubated with Grx2 antibody conjugated with Alex 594 red dye and Nrf2 antibody conjugated with Alex 488 dye, producing a merged yellow overlay. HepG2 cells were counterstained with DAPI to view the nuclei (blue). All three stains provided a distinct white hue, showing that Grx2 and Nrf2 interact in the nucleus.

Grx2 may be involved in DNA repair in the nucleus.

Because evidence shows that Grx2 may be abundant in the nucleus, a NucBlue live cell stain was performed to discover a possible function of Grx2 in the nucleus. As seen in fig. 24, scramble shRNA and Grx2 shRNA HepG2 cells have a bright blue nucleus due to cancer's high rate of DNA replication and synthesis [1]. However, upon doxorubicin treatment, Grx2 shRNA HepG2 cells are severely affected with no distinct or definitive morphology and a blurred image at both 5 uM and 10 uM doxorubicin treatment (Fig. 24). Scramble shRNA HepG2 cells lose some bright blue fluorescence but remain in perfectly round shape and normal morphology with doxorubicin treatment (Fig. 24).

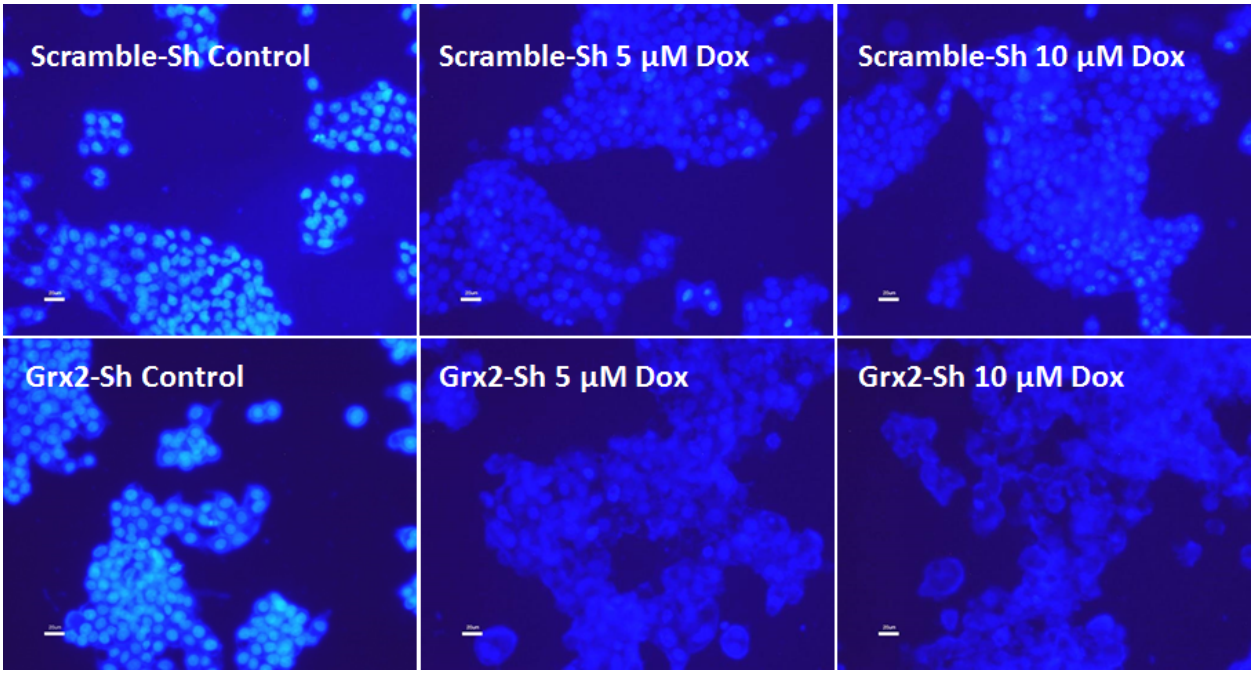

Fig 24. Grx2 may be involved in DNA repair in the nucleus. Scramble shRNA and Grx2 shRNA HepG2 cells with and without 5 uM and 10 uM doxorubicin treatment were stained with the NucBlue live cell stain and imaged using a confocal microscope.

Glutaredoxin inhibition decreases mRNA expression levels of Nrf2 pathway-related genes.

In order to determine future directions for this research and see whether there is an effect on the mRNA level, an antioxidant gene screening method using qPCR was performed. Scramble shRNA, Grx1 shRNA, and Grx2 shRNA were treated with doxorubicin for 6 h and then had RNA extracted. The RNA was then converted to cDNA using reverse transcriptase and then loaded onto a 96 well plate containing 48 Nrf2-related pathway genes. Scramble shRNA with doxorubicin treatment was used as a reference point for all gene expression changes.

The most significant gene changes respective to our work are displayed on figure 21. As shown, survival genes were the most impacted by glutaredoxin inhibition. Grx1 shRNA had nearly doubled in caspase-3 mRNA levels and a 1.2-1.3x increase in pro-apoptotic factors like Bax and caspase-7 compared to scramble shRNA with doxorubicin treatment (Fig. 25). Grx1 shRNA HepG2 also showed about a 70% decrease in Bcl2 level compared to control (Fig. 25). Grx2 shRNA with doxorubicin treatment showed similar results for survival genes mRNA expression level but was not affected as much in caspase-3 level. Caspase-3 mRNA levels were about 1.4 times as much as scramble shRNA with doxorubicin treatment (Fig. 25).

The dual oxidases, glutathione-S-transferases, and peroxiredoxin 6 are considered to be products of Nrf2-ARE activation. Dual oxidases have roles in cytokine signaling and inflammatory response as well as the regulation of ROS production [63]. Glutathione-S-transferases pi and mu are often linked to cancer drug resistance and are glutathione synthesis enzymes [64]. Peroxiredoxin 6 is a Nrf2-regulated enzyme that detoxifies peroxides using GSH as a cofactor [65]. Dual oxidases decreased about 55-65%, whereas the glutathione-S-transferase isoforms were decreased about 35-45% in Grx1 shRNA HepG2 group. The dual oxidases and glutathione-S-transferases were both heavily affected with a greater than 85% decrease in mRNA expression

level for Grx2 shRNA HepG2 group (Fig. 25). Peroxiredoxin 6 decreased 45% in Grx1 shRNA HepG2 group after doxorubicin treatment. Peroxiredoxin 6 was slightly more affected in Grx2 shRNA HepG2 group than Grx1 shRNA with doxorubicin treatment, decreasing approximately 60% compared to scramble shRNA with doxorubicin treatment (Fig. 25).

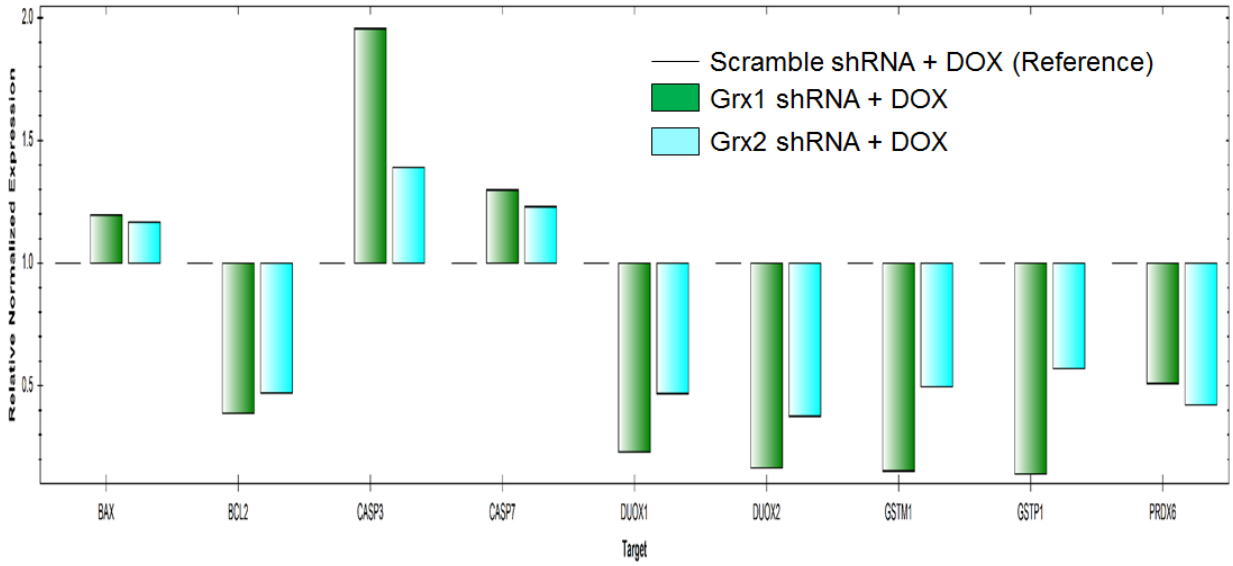

Fig 25. Grxs inhibition decreases the mRNA expression levels of Nrf2-related pathway genes. cDNA was extracted from scramble shRNA, Grx1 shRNA, and Grx2 shRNA HepG2 groups treated with 10 uM doxorubicin for 6 h and tested on an antioxidant enzyme pathway panel qPCR assay for 91 Nrf2-pathway related genes. The most significantly impacted genes are presented in this graphical depiction including Bax, Bcl2, caspase-3 (CASP3), caspase-7 (CASP7), dual oxidases 1 and 2 (DUOX1 and DUOX2), glutathione-S-transferases (GSTM1 and GSTP1), and peroxiredoxin 6 (PRDX6). Quantification of fold changes were normalized to GAPDH mRNA and then referenced to scramble shRNA with doxorubicin as baseline (n=1).

DISCUSSION

Cancer drug resistance due to the overexpression of antioxidant enzymes is becoming an increasingly bigger problem. In particular, hepatocellular carcinoma is known to be less sensitive to anti-cancer regiments with chemotherapy and surgery only successful in 10-20% of patients [2]. With a huge morbidity rate, hepatocellular carcinoma remains an elusive problem to oncologists and many scientific researchers. Moreover, with the possibilities of remission in cancer and cancer metastasis, using higher concentrations of anti-cancer drugs has only seen enhanced drug toxicity, life threating side effects, and even patient death [9, 43]. Despite new advancements supporting other antioxidant inhibitors, finding an antioxidant target that can help kill cells and sensitize cancer cells to anti-cancer drugs is a challenge. One key antioxidant system, the glutaredoxin system, is primarily responsible for cleaving PSSG, yet the latest research has shown that glutaredoxin has multiple other functions involved in the apoptotic pathway and cellular protection [16]. Glutaredoxin is vital for cell viability and growth and operates fairly independently within the cell. However, glutaredoxin's expression and function in cancer especially hepatocellular carcinoma is still relatively unknown.

Findings from our preliminary clinical study showed that glutaredoxin is overexpressed in liver cancer. Glutaredoxin overexpression is a common moiety in notorious drug-resistant cancers such as MC7 breast cancer and small cell carcinoma [57]. Studies by Lillig et al in 2004 inspired the inhibition of Grx2 using siRNA as a method to increase doxorubicin-induced apoptosis in HeLa cells [18]. Although his research clearly defined glutaredoxin as a potential anti-cancer target, he failed to define the mechanism behind the increased apoptosis and why glutaredoxin on a molecular basis would make an ideal target. The key purpose of this study then was to

comprehend the effects of glutaredoxin inhibition on apoptosis and doxorubicin sensitivity in hepatocellular carcinoma and explore the molecular basis behind this theory.

To obtain the glutaredoxin knockdown cell lines, Grx1 and Grx2 shRNA was used and caused 50-70% knockdown (Fig. 5 and Fig. 6). shRNA was used because it is known to be more longer lasting than siRNA. Moreover, there is currently no efficient and non-toxic chemical inhibitors available [66]. After obtaining efficient knockdown, we used these cell lines in an apoptotic study to substantiate our claim that glutaredoxin inhibition would promote doxorubicin sensitivity. From the WST-8 assay (Fig. 7), we could clearly see that glutaredoxin inhibition decreases cell viability especially for 1 and 10 uM doxorubicin treatments. This was further collaborated by morphological data showing much fewer cell numbers for the Grx1 and Grx2 shRNA HepG2 groups. Apoptotic pathway proteins are more obvious markers of cell death, so crucial apoptotic proteins like Bax, Bcl2, and cleaved caspase-3 were used to mark the different stages of cell death. This supports studies showing that Bcl2 is regulated by Grx1 and that cleavage of caspase-3 may also be under glutaredoxin regulation [24, 25]. With glutaredoxin inhibition, Bcl2 is clearly decreased even without doxorubicin treatment in glutaredoxin inhibited cells, whereas Bax surprisingly upregulated over three times as much in Grx2 inhibited cells. Cleaved caspase-3 similarly upregulated nearly twice as much after doxorubicin treatment for Grx1 and Grx2 inhibited cells. PSSG accumulation is also an important indicator of cell death. PSSG may alter cell function, especially since glutathionylation affects cysteine residues that are often localized to the active sites of proteins [67]. PSSG accumulation also corresponds with abundant oxidative stress and thus usually inactivates crucial enzymes. Glutaredoxin normally is present to protect the cells from PSSG formation by cleaving PSSG, but as seen in fig. 10 and 11, PSSG accumulation and actin glutathionylation significantly increased with glutaredoxin inhibition and

doxorubicin treatment. This emphasizes the importance of glutaredoxin in maintaining a good redox state in the cell and how glutaredoxin inhibition can ultimately increase oxidative stress and prevent cancer metastasis. All this evidence promotes the idea that doxorubicin sensitivity increases with glutaredoxin inhibition.

However, the cell signaling pathways that enabled glutaredoxin inhibition to increase doxorubicin sensitivity was still a mystery. Doxorubicin is unique in its ability to generate ROS, create oxidative stress and thus cause cell death [5]. Oxidative stress often leads to the glutathionylation of proteins [67]. The cysteines located in transcription factors' active sites are subject to post-translational modifications like glutathionylation that can promote or decrease DNA binding activity. Nonetheless, reversible glutathionylation mediated by Grx can allow for deglutathionylation of crucial proteins in oxidative stress to enhance cell survival. Like other antioxidant enzymes, glutaredoxin is known to be regulated by Nrf2. However, more studies support bidirectional feedback where certain amino residues on transcription factors can be regulated by their downstream targets [68]. The plasticity of the cell defense system underlies the innovation of how downstream or phase II proteins can also regulate their transcription factors, bringing up the question of whether glutaredoxin can facilitate Nrf2 enzymatic activity. Although most studies support that disruption of Keap1's cysteine residues promote Nrf2 activation, He and Ma provided a novel theory highlighting Nrf2's cysteine residues as more important redox sensors and that Keap1 dissociation was not enough. Specifically, mutated or hindered Nrf2's cysteine residues would prevent Nrf2 activation [54]. As such, Nrf2's cysteine residues are prone to post-translational modifications that would allow for the activation and translocation of Nrf2 to the nucleus. He and Ma already highlighted the importance of phosphorylation of Nrf2 as a method of increasing the trafficking speed of Nrf2 into the nucleus, but Nrf2 regulation via S-

glutathionylation has still not been studied [51]. The relationship between Nrf2 and glutaredoxin expression clearly showed a definite correlation, as glutaredoxin inhibition lead to Nrf2 decrease especially after doxorubicin treatment (Fig. 12). The substantial decrease in Nrf2 prompted us to see whether Nrf2's downstream targets were also affected. NQO1, catalase, and HO-1 were significantly decreased especially after doxorubicin treatment in Grx1 and Grx2 inhibited groups compared to scramble shRNA due to Nrf2 decrease (Fig. 13, Fig. 14, and Fig. 15). Trx1 and Trx2 showed an interesting relationship with glutaredoxin inhibition. Because thioredoxin and glutaredoxin often cross-talk and are reinforcement systems of each other, glutaredoxin inhibition promptly caused a thioredoxin increase, but in oxidative stress conditions, thioredoxin decreases to baseline levels because of the sparse amount of Nrf2 present in the cell (Fig. 16 and Fig. 17). Our antioxidant gene based screening method also showed interesting mRNA levels of Nrf2-related pathway proteins. Particularly, the survival genes were most affected with severe decreases in pro-apoptotic proteins and anti-apoptotic proteins in glutaredoxin inhibited cells (Fig. 25). This is to be expected since it is well known that glutaredoxin regulates caspase-3 and Bcl2 levels. Moreover, since Bcl2 controls Bax expression, Bax would be increased without Bcl2 inhibition [26]. Interestingly, glutathione synthesis enzymes, which are downstream Nrf2 targets, were also affected. Glutathione-S-transferase pi and mu isoforms were downregulated in Grx-inhibited cells and is the second step of glutathione synthesis (Fig. 25). Without glutathione synthesis, glutaredoxin would be unable to perform its job, but GSH is a vital cofactor that allows for oxidized glutaredoxin to return back to its reduced active state [16]. Moreover, glutathione-S-transferase pi overexpression has been linked to cancer drug resistance and detoxification, as the absence or decrease of glutathione-S-transferase mu isoform often corresponds with carcinogenesis and susceptibility to bladder, colon, skin, and possibly, lung cancer [64, 66]. Consequently,

peroxiredoxin 6, a Nrf2 regulated enzyme, and dual oxidases 1 and 2 which are heavily involved in scavenging free radicals and promoting cell survival were also decreased in Grx1 and Grx2 shRNA HepG2 cells. Peroxiredoxin 6 is unique compared to other peroxiredoxins in that it uses GSH as a cofactor and not thioredoxin to directly reduce peroxides. It is also the only peroxiredoxin with the capacity to reduce phospholipids as well, so it is heavily involved in repairing cellular membranes damaged by oxidative stress [65]. The decrease in GSH synthesis enzymes and Nrf2 may have contributed to the decrease in peroxiredoxin 6 expression. The other tested enzymes were not as affected. This could have been a problem with the 6 hour incubation time to obtain the mRNA samples or the older generations of HepG2 used to complete this experiment. Overall, the evidence suggests that Nrf2 and its downstream proteins are greatly impaired by glutaredoxin inhibition.

Glutaredoxin's substrates are glutathionylated proteins, and because Nrf2 has cysteine residues that can act as redox sensors, Nrf2 also has the potential to be glutathionylated via post-translational modifications. However, glutathionylation is often linked to inactivation of enzymes because glutathionylation can hinder cysteine residues that are part of the active sites of proteins [68]. Because of this, it was hypothesized that Nrf2 could be a substrate for glutaredoxin and that increased Nrf2 glutathionylation would lead to inactivation or degradation of Nrf2. If glutaredoxin is inhibited and oxidative stress conditions exist, there would not be enough glutaredoxin to deglutathionylate and activate Nrf2 to transcribe vital antioxidant enzymes that can protect the cancer cell, resulting in increased cell death and doxorubicin sensitivity (Fig. 3). To validate this statement, it was necessary to see the binding interaction between Grx1 and Grx2 with Nrf2. As seen in fig. 18, Grx1 and Grx2 binding to Nrf2 significantly increased two-fold. Also, Nrf2 glutathionylation increased with glutaredoxin inhibition, whereas Nrf2 glutathionylation

significantly decreased in scramble shRNA HepG2 after doxorubicin treatment, indicating increased Nrf2 activation and trafficking into the nucleus (Fig. 19, Fig. 20, and Fig. 21). With the Nrf2 and its downstream antioxidant proteins decrease, this showed that glutaredoxin inhibition enhanced the impairment of the Nrf2-dependent antioxidant response.

However, understanding the fate of Nrf2 after glutathionylation was complicated. Despite promising results in our whole cell lysate, it was important to see if Nrf2's translocation to the nucleus was hindered due to degradation. Moreover, Grx1-Nrf2 binding could be explained because Nrf2 is found in both the cytoplasm and the nucleus, but Grx2, a mitochondrial antioxidant, binding with Nrf2 was still unclear unless Grx2 exerts its protective effects in the nucleus as well. Despite cloning of Grx2 in the nucleus, there is no confirmed evidence of Grx2's presence in the nucleus [17]. Moreover, another study performed in 2010 highlighted that Grx2b and Grx2c transcripts may be overexpressed in certain cancer cell lines in both the cytoplasm and the nucleus despite that these isoforms were once considered testes-restricted [19]. Interestingly, Grx1 and Grx2 was found in the nucleus, but because of possible cytoplasmic contamination, it is more likely that Grx2 is a nuclear resident (Fig. 21). Moreover, Grx2 is found in high amounts in scramble shRNA and increased with doxorubicin treatment, but Grx2 shRNA HepG2 group had nonexistent Grx2 levels after doxorubicin treatment. This could validate the low expression of Nrf2 in Grx2 inhibited cells and also show how Grx2's nuclear location could impact Nrf2's transcription of antioxidant enzymes (Fig. 12 – Fig. 17). However, the effect on Nrf2 expression was not as extreme as previously seen. This might have been contributed to 6 h incubation time. Future studies will have to address whether Nrf2 continues to decrease at more time points with doxorubicin treatment. Even if Nrf2 is still able to make it in its glutathionylated state into the nucleus, Grx2 would be practically nonexistent in the nucleus to activate Nrf2, causing no

transcription of ARE (Fig. 21). Moreover, Grx2 could play an important role in continuous activation of Nrf2 for transcription of antioxidant proteins to compensate for the continued stress environment in cancer cells. Our immunostaining data clearly shows that Nrf2 and Grx2 interact within the nucleus, so it is highly likely that Grx2 catalyzes Nrf2 repair as well as many other transcription factors with important redox sensors (Fig. 22 and Fig. 23). Considering doxorubicin's ROS production is highly concentrated in the nucleus, this might explain why Nrf2 inactivation may be accelerated by Grx2 inhibition [10].

However, the function of Grx2 in the nucleus is still virgin territory. Our preliminary data using NucBlue live cell staining highly suggests that Grx2 may be crucial to DNA repair and synthesis in the nucleus (Fig. 24). Grx2 shRNA HepG2 cells after doxorubicin treatment did not have any definite morphology with significantly less DNA compared to control (Fig. 24). Moreover, glutaredoxin system was primarily discovered as an electron donor to ribonucleotide reductase, an enzyme heavily involved in the production of dNDPs. Without dNDPs available, genome and mitochondrial DNA synthesis and repair would be significantly impaired since ribonucleotide reductase is the DNA synthesis rate-limiting reaction [70]. The ideal location of Grx2 in the nucleus may give cancer cells a direct advantage in DNA synthesis and repair and may promote resistance to DNA-targeting cancer drugs such as intercalating agents like doxorubicin and DNA synthesis inhibitors. The discovery of Grx2 in the nucleus could open a lot of future research studies on the function of Grx2 in the nucleus especially Grx2's effect on crucial transcription factors.

CONCLUSION & FUTURE STUDIES

This study explored the effects of glutaredoxin inhibition on doxorubicin sensitivity and the Nrf2-dependent antioxidant response in hepatocellular carcinoma. The data we obtained strongly allege that glutaredoxin is a strong contender as a pharmaceutical target to both increase cancer cell death and sensitize cancer cells to doxorubicin. Glutaredoxin's multiple antioxidant functions including regulating survival genes like Bcl2, peroxidase activity, and actin polymerization make glutaredoxin a vital defense enzyme in cancer. With glutaredoxin inhibition, the Nrf2-dependent antioxidant response is heavily impaired, causing an abundance of oxidative stress that can lead to cancer cell death and ultimately prevent cancer metastasis as well.

Future studies concentrating on the effectiveness of Grx inhibition on a drug-resistant hepatocellular carcinoma and other cancer cell lines can further elucidate the function of glutaredoxin in cancer. Some studies have already emphasized how Grx and Nrf2 are involved in the regulation of the 20S proteasome for the degradation of oxidized proteins and even Nrf2 [35, 71]. Looking more into the ubiquitination and degradation of Nrf2 may provide more insight into how Grx and Nrf2 are abundant in cancer, assist in less drug sensitivity, and may regulate each other. Moreover, it would be imperative to explore the impact of Grx inhibition on other survival pathways involved in cancer such as NFkB, AP-1, and AKT. Considering our discovery that Grx2 is in the nucleus, it is possible that Grx2 is involved in regulating many other transcription factors, so determining Grx2's nuclear targets is another direction our future research hopes to take. Finally, finding a more efficient way to administer Grx shRNA particles either through nanoparticles or immune cell vectors or discovering nontoxic Grx inhibitors can revolutionize anti-cancer therapies and hopefully make it a paramount treatment for cancer patients.

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