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The role of NAD(P)H:quinone oxidoreductase 1 in non-small cell lung cancer tumorigenesis and lung cancer stem cell maintenance

Brian Madajewski

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**The role of NAD(P)H:quinone oxidoreductase 1 in non-small cell lung cancer
tumorigenesis and lung cancer stem cell maintenance**

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PhD Dissertation submitted
to the West Virginia University School of Medicine
at West Virginia University
in partial fulfillment for the degree of

Doctor of Philosophy
in
Cancer Cell Biology

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resistance,

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Abstract

The role of NAD(P)H:quinone oxidoreductase 1 in non-small cell lung cancer tumorigenesis and lung cancer stem cell maintenance

Brian Madajewski

Lung cancer is the leading cause of cancer related deaths worldwide. The five-year survival rates for those patients suffering from non-small cell lung cancer (NSCLC), continues to be an abysmal 15%. One of the major reasons for the poor survival rate amongst NSCLC patients is the lack of early detection and subsequent late stage initial diagnosis. Tumors discovered at later stages are often refractory toward chemotherapy and radiation regimens. One theory as to why tumors become resistant to therapy relies heavily on the cells that make up the cancer stem cell (CSC) niche. This small percentage of cells within the heterogeneous tumor has been reported to be responsible for drug resistance, tumor recurrence, and metastasis. In general, CSCs have been isolated using a number of different markers, including cluster differentiation markers, somatic stem cell markers, as well as a number of functional markers such as the side population and aldehyde dehydrogenase (ALDH) activity. While some cancer types, such as breast and hematologic cancers, have been significantly investigated to identify and define their CSC population, lung cancer researchers have only recently begun to identify CSC markers in lung tumors. In addition to the CSC population, malignant cells can also alter their expression of a number of cytoprotective genes that promote tumorigenesis. NAD(P)H:quinone oxidoreductase 1 (*NQO1*) is a detoxifying enzyme that has been demonstrated to be highly overexpressed in a number of different malignancies. This overexpression has been utilized as a drug target, as the enzyme is expressed at low levels in normal tissue. To this point, there has been success in using *NQO1* as a drug target, however little research has been conducted on understanding why *NQO1* is overexpressed in these malignancies. The work presented here investigated the role of *NQO1* in tumorigenesis as well as its role in maintaining the CSC population in NSCLC. We demonstrate that *NQO1* promotes anchorage-independent growth, invasion, reactive oxygen species regulation, anoikis resistance, proliferation, *in vivo* tumor growth, survival, and ALDH activity. Secondly, we demonstrate that *NQO1* also promotes spheroid formation, both in initial and serial contexts, enhances the CSC frequency, and protects spheroid-cultured cells from chemotherapy. Finally, we provide preliminary data that indicates that *NQO1* mRNA may be playing an important signaling role in the promotion of the CSC phenotype. This was demonstrated by CRISPR-Cas9 genetic knockout of *NQO1* that resulted in a reemergence of the CSC phenotype that can be reversed with transient knockdown of *NQO1* mRNA. In summary, our data demonstrate that *NQO1* is playing a vital role in the promotion of NSCLC tumorigenesis, as well as supporting the cancer stem cell population. Interestingly, these results may be due to a novel signaling mechanism by *NQO1* mRNA, and not the enzyme itself. Further research will be needed to completely understand the role of *NQO1* mRNA in NSCLC tumorigenesis and the CSC phenotype.

Dedication

This dissertation is dedicated to my parents, Cheryl and David Madajewski, whom have instilled in me the characteristics of hard work and determination. These traits have allowed me to reach my goals both academically, and in life. It would have been impossible to achieve this milestone without their help. Their undying love and support has helped turn my dream into reality, and for this I am forever grateful. I also want to thank my sister, Amanda Madajewski, for her unwavering support and candid reality checks throughout this process. She has always helped to remind me of the bigger picture, and has played an integral part in propelling me through my doctoral work.

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Chapter 1

Introduction and Literature Review

Introduction

Lung cancer is the leading cause of cancer related death in the world (1). It is predicted to be responsible for more than 158,000 deaths in the United States in the year 2015 alone (2). In addition, the disease claims a greater number of lives than breast, prostate, and colon cancer combined. Lung cancer is classified into two histologically different categories, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC arises from a neuroendocrine origin, and occurs at a rate of 20% of all lung malignancies. These tumors are often more aggressive than their NSCLC counterparts, resulting in 5-year survival rates of less than 5% versus 15% seen in NSCLC (3, 4). The survival rates vary greatly depending on the stage at which the cancer is detected, but often patients with SCLC present with advanced and disseminated disease leading to an overall increase in mortality (5).

NSCLC is the most common type of lung cancer in the world, accounting for ~80% of all lung cancer cases (6). NSCLC arises from the epithelial cell population within the lung and can be further divided into three subcategories based on histological assessment. Areas of keratinization and the presence of inflammatory components define the first subtype of NSCLC, squamous cell carcinoma. Adenocarcinoma is defined by the presence of glandular structures, or solid growths exhibiting mucin production. This subtype also tends to be found in the periphery of the lung, where squamous cell carcinoma typically originates near the bronchus. The histological distinction of the third NSCLC subtype, large cell carcinoma, is decidedly more vague. Cases lacking the defining features of squamous cell carcinoma or adenocarcinoma tend to be classified as large cell carcinoma (7, 8). While it is possible to histologically differentiate each tumor type, these distinctions provide little information on the likelihood of recurrence, drug resistance, and metastatic capabilities of the cells. Currently, it has become an area of great interest to determine the overall cellular makeup of each tumor and define certain niches of cells that exist within, in order to improve therapeutic approaches and patient outcomes.

Recently, the nearly 50-year-old hypothesis that cancer arises from a small population of tumor initiating, or cancer stem cells (CSCs) has begun to once again grab the attention of researchers (9). The belief is based on the premise of somatic stem cells and their unique abilities. It is well known that somatic stem cells are crucial for growth, differentiation, and repair of the normal tissues. They achieve these affects by providing an unending supply of progenitor cells, while retaining the parental stem cell population through asymmetric division (10). Adopting these traits, it is now believed that cancers are capable of sustaining growth and promoting recurrence due to the presence of a cancer stem cell (CSC) population. These cells harbor the ability to self-

renew and give rise to progeny that are then able to differentiate into all cell types necessary to constitute the complete makeup of a tumor.

Interestingly, it has also been demonstrated there exists an inherent plasticity in transformed cells. Differentiated cancer cells possess the ability to return to their less differentiated CSC phenotype as well revert back, thus indicating a divergence from regulated somatic cell differentiation (11, 12). Nevertheless, this CSC population appears to mediate resistance to therapy, making them difficult to eliminate (13-15). This poses an obvious challenge in the treatment of cancer and in fact, therapies have been shown to select for the more aggressive CSC phenotype, through the elimination of the non-stem cell population (16-18). In addition to therapeutic resistance, the CSC population has displayed enhanced capabilities in the processes of degradation, invasion, and metastasis in comparison to their non-stem cell counterparts present within a malignancy (14, 19, 20).

Due to CSCs abilities to reconstitute the entire heterogeneous makeup of a tumor, their resistance to standard therapies, and demonstration of an increase in the ability to metastasize to distant sites, a great amount of research has been directed at defining this population in NSCLC. Elucidating this population will have great implications in both the diagnostic and therapeutic aspects of cancer therapy. However, this approach is made difficult by the elusiveness of the CSC population within the heterogeneous population, since they can constitute as little as five percent of the total cell population. This percentage however, can vary depending on the selection marker or method (21). Currently, research is focused on a number characteristics thought to be correlative to the CSC population, including specific cluster differentiation (CD) markers, efflux pumps, detoxifying mechanisms, and stem cell signaling pathways. In order to understand the importance of the cancer stem cell population and its implications to prognosis, therapeutic resistance, recurrence, and the future of drug design, it is first necessary to understand the concept of the cancer stem cell hypothesis and those markers used to define this elusive population.

The Cancer Stem Cell Hypothesis

The cancer stem cell hypothesis originated more than 50 years ago, and stated that a small subset of cells within the overall tumor population were responsible for the initiation, propagation, and maintenance of the neoplasm (22). In order for this to be true, the CSC

Figure 1. Symmetric and asymmetric division of stem cells. Stem cells (orange cells) are able to divide either asymmetrically into progenitor cells (pale blue cells), or into another stem cell through symmetric division. The symmetric divisions allow stem cells to continually repopulate the stem cell pool, whereas asymmetric division will give rise to progenitor cells. The progenitor cells then begin to differentiate in multipotent, early progenitor cells (pink cells) that will finally give rise to fully differentiated cell types (multi-colored cells) that make up the respective tissue.

population would need to display two characteristics that were already demonstrated by the somatic stem cell population, 1) self-renewal and 2) the ability to give rise to differentiated cell types (**Figure 1**) (9). By satisfying both of these requirements, CSCs would be able to maintain the necessary stem cell population, as well as produce the heterogeneous assortment of cells seen in malignancies.

The belief in CSCs was bolstered when Dick and colleagues were able to isolate a CSC population within leukemia. Their work was the initial demonstration that a single malignant cell could be administered *in vivo* and result in the onset of leukemia. The induction of leukemia was capable of being carried out in serial experiments by isolating the CSCs, and re-injecting that population into subsequent animals. The very first CSC marker definition that they utilized was $CD34^{+}/CD38^{-}$ (22).

Due to the impact of this finding, the cancer research community began to investigate the possible presence of CSCs in all other forms of cancer. Since that time, CSCs have been defined in multiple cancer types including breast, prostate, pancreas, brain, ovary, and colorectal (23-28). However, this population has yet to be exhaustively defined in NSCLC.

Lung Cancer Stem Cells

In the search for lung CSCs, researchers have developed a set of criteria in order to delineate the stem cell population from that of the bulk tumor. The majority of these characteristics have been adopted from the traits that normal, somatic stem cells display. This approach has been utilized to define the CSC population throughout numerous cancer types, and is being adopted similarly in lung cancer (10). The required traits are commonly evaluated based on *in vitro* and *in vivo* assays, with the latter approach providing the most definitive demonstration of a CSC. These approaches and the traits they assess are briefly explained below.

In vitro Cancer Stem Cell Assays

There are a number of popular *in vitro* studies being utilized to demonstrate the difference in the prominence of the CSC phenotype between various cell populations. These techniques include sphere formation, soft agar colony formation, collagen invasion, matrix degradation studies, and chemotherapeutic resistance assays (14, 29-32). These assays demonstrate necessary characteristics of CSCs. Examples of this include the ability to grow in an anchorage independent manner. The ability to grow in the absence of a matrix is a hallmark of transformed cells. Spheroid formation and soft agar colony formation are both assays designed to evaluate a cell's ability to proliferate in the absence of matrix attachment (33). Additionally, spheroid culture techniques utilizing restricted media formulations are often implemented to specifically enhance CSC populations. The reduction of differentiation-inducing factors allows for the expansion of the undifferentiated CSC population (34, 35).

The ability of malignant cells to degrade matrix and invade into the surrounding tissue are two necessary processes of metastasis (36, 37). Interestingly the emergence of metastatic disease is associated with increased mortality, where 90% of cancer deaths are caused by metastases (38). The ability to establish metastatic disease has been connected to the CSC population (19, 39). Migration and invasion capabilities are often evaluated using a number of assays including wound healing, trans-well migration, spheroid invasion, and organotypic cultures that more closely resemble the *in vivo* environment (40, 41). Increases in these characteristics are often observed in the CSC population in comparison to the non-CSC population, demonstrating a more invasive phenotype (42).

Finally, examining the therapeutic resistance of a CSC population demonstrates the inherent trait of being unaffected by cytotoxic therapy. Malignant cells that are resistant to both chemotherapeutic and radiation treatments can lead to the recurrence of cancer following significant reduction of the disease due to bulk tumor susceptibility to these approaches (43). The resistance of CSCs to therapeutic approaches is so robust that often CSCs are often selected for by treatment with chemotherapeutic drugs or radiation (13). These results demonstrate the difficulty faced when trying to eliminate this population, and demonstrate that treatments spare CSCs in comparison to the overall tumor cell population (44).

In an effort to enhance the CSC population, and reduce the presence of non-CSCs, these described techniques tend to utilize cells that are cultured as spheroids. The ability to survive and expand in spheroid culture has been demonstrated to enhance the stem cell signature in both cancer and normal cells (45, 46). Thus, it makes utilizing spheroid culture an adequate method by which to expand the desired population for use in subsequent experimentation.

In vivo Cancer Stem Cell Assays

A hallmark of a CSC population is increased tumorigenic propensity in comparison to bulk tumor populations. Demonstration of increased tumor initiating capabilities can be exhibited by injecting far fewer numbers of CSCs into immunocompromised animals, in comparison to heterogeneous cell populations. The results demonstrate that injections with reduced CSC numbers continually lead to tumor engraftment and growth that resemble results observed with utilizing significantly greater numbers of non-CSC (47). This is known as the *in vivo* limiting dilution assay, which continues to be held as the gold standard demonstration of a CSC population. Tumors that arise can then be assayed for resistance to therapy, invasiveness, and metastasis to sites such as the lymph nodes, liver, bone, and brain. (48-51).

Self-renewal of the CSC population is also evaluated *in vivo* by performing serial tumor forming assays in immunocompromised mice. Much like serial spheroid formation assays *in vitro* evaluate a population's ability to continually replenish the CSC population, so do serial tumor forming assays (10). Continued tumor formation following dissociation of primary tumors and injection into secondary recipients demonstrates the

population's ability to continually produce the tumor initiating, CSC population. Further serial injections are often performed to validate the self-renewal capabilities of the population in question (52).

The assays described above provide a functional validation of the CSC phenotype, however they do not provide a suitable definition of the population of cells of interest. In order to be able to effectively isolate and identify the CSC population it is commonplace to select cells based on markers, often cluster of differentiation (CD) markers are utilized. These markers are present on the surfaces of cells, and have been utilized to isolate cell types depending on their specific CD marker expression (53). In the search for CSCs, many investigators have coopted CD markers from other cancer types to investigate their potential as a useful identifier of CSCs within their cancer of interest. In addition to CD markers, the utilization of hyperactive signaling cascades can also be utilized to define a CSC population.

Currently, NSCLC research is at the stage of utilizing known CSC markers from other defined CSC populations like those found in breast, prostate, and glioblastoma in an effort to isolate the NSCLC CSC population. Below, we review the most commonly implemented markers and signaling pathways used in the attempt to define the NSCLC stem cell population. **(Figure 2)** It should be noted that this field is currently in its infancy, and there exist a large number of conflicting reports that make it very difficult to support the utilization of many of these CSC markers in NSCLC.

Figure 2. CD markers and cellular detoxification in non-small cell lung cancer. Cancer stem cell populations are commonly separated based on CD markers or detoxification pathways. Common CD markers utilized in determining the non-small cell lung cancer population include *CD133* and *CD44*. *CD133*, a five-transmembrane receptor, currently has no known function. *CD44*, the hyaluronic acid receptor, has been utilized as a popular CSC marker in a number of malignancies. ABC pumps are responsible for the characterization of the side population. Cells that express the ABC transporters are capable of extruding Hoechst dye, and those cells with low dye retention are classified as the stem cell population. Aldehyde dehydrogenase enzyme activity has been utilized across many cancer types to define the CSC population. Cells with high aldehyde dehydrogenase activity, as defined by the Aldefluor Kit, have been demonstrated to have increased CSC characteristics compared to those with low aldehyde dehydrogenase activity.

Cluster Differentiation Markers

CD133

CD133, also known as Prominin 1, is a five transmembrane glycoprotein initially believed to be a hematopoietic and neural stem cell marker in humans (54, 55). It has since been confirmed to be a hematopoietic stem cell marker, and is utilized in the screening of blood cells for allogenic transplantation (56). *CD133* has also been employed to identify the stem cell population within various types of malignancies including colorectal, brain, gastric, and NSCLC (57-60). Cells that are determined to be *CD133*⁺ within a tumor have been reported to demonstrate the characteristics associated with CSCs and function as prognostic indicators (57, 61). While this CSC marker is believed to be highly correlative to the

CSC phenotype, there are a number of conflicting reports concerning its association with stemness. An example of this can be seen in the opposing findings produced by Tirino and Meng *et al* (5, 62).

Tirino and colleagues have shown *CD133* to be a strong marker of stemness, selecting for cells with increased abilities to form spheres, increased levels of anchorage-independent colony formation, and increased tumorigenic potential *in vivo*. However, Meng and colleagues refute these findings by reporting that both *CD133*⁺ and *CD133*⁻ tumor cells are capable of forming spheres, as well as give rise to colonies in soft agar experiments. Meng *et al.* also reported that *CD133*⁺ and *CD133*⁻ cells were both capable of *in vivo* tumorigenesis, and conveyed no discernable difference in their invasiveness or resistance to chemotherapeutics.

While the studies referenced above demonstrate a difference in opinion, they are not the only ones to do so. A large amount of literature can be found either for or against the concept that *CD133* expression can be utilized as a reliable marker to define the CSC population within a tumor (57, 63, 64). Currently, it is clear that more research must be done to determine if *CD133* can be utilized as a definitive marker for CSC within a heterogeneous tumor population.

CD44

CD44, like *CD133* is an integral membrane glycoprotein expressed on the surface of cells, and functions as the hyaluronic acid (HA) receptor (65). *CD44* is most well known for its role as a CSC marker in both breast and prostate cancers. CSC populations in these tumors are defined as *CD44*⁺/*CD24*^{-/low}, and it is this population that is said to harbor the necessary traits to be defined as CSCs (23, 66). Currently, the usage of *CD44* in lung cancer is not well studied. However, in accordance with implementing known CSC markers from other cancer types, researchers are beginning to explore the possibility of utilizing *CD44* as a NSCLC stem cell marker. In one study, Leung and colleagues discovered that *CD44*⁺ NSCLC cells were enriched in stem cell properties, but this finding was only applicable to their large cell carcinoma model. The stem cell characteristics of *CD44*⁺ cells collected from an adenocarcinoma model failed to demonstrate the increased tumorigenicity or drug resistance, seen in the LCC model (67).

In agreement with the findings mentioned above, Wang *et al.* demonstrated that *CD44*⁺ cells collected from NSCLC also had increased stem cell properties, however this study also utilized the secondary marker, *CD90*, to further select the cell population of interest. It was observed that upon collection of *CD44*⁺ cells, the properties displayed by CSCs increased in this population. The phenotype was further enriched upon secondary selection with *CD90* (68). The subsequent *CD44*⁺/*CD90*⁺ classification calls into question the value of *CD44* as a standalone CSC marker in NSCLC. It is difficult to say that the population collected by *CD44* alone is the stem cell population due to *CD90* increasing the CSC characteristics further. This study lacked the control of utilizing *CD90* alone as a CSC marker. Recently, *CD90* has been shown to define the CSC

population in esophageal cancer, further calling into question the applicability of *CD44* as a CSC marker in tumors that have yet to have a defined CSC population (69).

Overall, *CD44* has functioned extremely well as a CSC marker for both prostate and breast cancer. However, in NSCLC there is still little evidence to support the use of *CD44* as a CSC marker. Undoubtedly the completion of future work will elucidate whether *CD44* is a plausible option for selecting NSCLC stem cell populations.

Detoxification Pathways

Aldehyde Dehydrogenase (ALDH)

Aldehyde dehydrogenases are a family of enzymes responsible for detoxification, drug resistance and cell differentiation. Their role in normal cellular metabolism is the oxidation of harmful aldehydes into carboxylic acids, and the conversion of retinol into retinoic acid (70, 71). Tumor cells that are positive for ALDH (ALDH^{high}), and cells that are negative (ALDH^{low}) are often selected utilizing the Aldefluor kit from Stem Cell Technologies. This kit enables the labeling of cells with high levels of ALDH activity, and allows sorting utilizing fluorescence assisted cell sorting (FACS) analysis.

In the lung epithelia, the expression and induction of isoforms *ALDH1A1* and *ALDH3A1* have been thoroughly investigated. It has been shown that the expression of both isoforms increase during malignant transformation of normal lung tissue (72, 73). Other studies have also demonstrated an increased expression of *ALDH1A1* and *ALDH3A1* in NSCLC cell lines (74). Interestingly, it was also observed that the expression of ALDH is higher in the NSCLC than the expression detected in SCLC leading to the possibility that ALDH expression may be a specific marker for NSCLC CSCs (72).

These initial studies provided some support that the activity of ALDH isoforms may be involved in NSCLC tumor initiation, progression, and stem cell maintenance. In subsequent studies conducted by Sullivan and colleagues (75), it was found that NSCLC cells that were found to be *ALDH1A1*⁺ had increased tumorigenicity, clonogenicity, and self-renewal capabilities in comparison to *ALDH1A1*⁻ controls. These findings were further supported by the correlation of poor clinical outcome in patients harboring *ALDH*⁺ tumors. Furthermore, this study demonstrated inhibition of the Notch signaling pathway greatly decreased the levels of ALDH⁺ cells, as well as their CSC characteristics.

In the Sullivan *et al.* studies discussed above, a controversial overlap between two purported CSC markers is highlighted. It has been suggested that one CSC marker, Notch, is responsible for the expression of another CSC marker, ALDH. In addition to the overlap, this study also disputed the previously discussed CSC marker *CD133*. In the manuscript by Sullivan and colleagues, *CD133* did not correlate with poor patient prognosis, indicating that it may not function as reliable prognostic tool. Additionally, these results demonstrate that, in some instances, CSC markers believed to be

perpetuating the CSC phenotype on their own may be working in conjunction with other CSC markers.

Side Population

The side population of tumor cells, is defined as those cells that are capable of efflux of the Hoechst 33342 dye (76). The ability of cells to have a high efflux capacity has been demonstrated in cancer stem cell populations, thus it can be utilized as a marker for the CSC population (77). Stem cells, and CSCs alike are able to efflux the dye through an up regulation of the ATP-binding cassette (ABC) family of transporters (78). These proteins lie within the membrane of cells and are responsible for the export of endogenous entities, as well as the expulsion of cytotoxic compounds used to treat malignancies (17).

It has been demonstrated repeatedly that the SP is capable of increased colony formation, tumor sphere formation, invasion, and drug efflux (17, 20, 79-81). Taken together, these characteristics provide a strong basis for characterizing these cells as a CSC population. However, it has also been demonstrated that cells making up the non-SP population are capable of giving rise to a SP. Additionally, the SP was also able to convert to other well-studied CSC such as $CD133^+$ and $ALDH^{\text{high}}$ cells (82). Findings such as this make it difficult to state whether a single marker is sufficient for defining the CSC niche, or perhaps that these cells are able to transiently express certain markers depending on cellular stage or state of tumor progression.

Signaling Pathways

Notch Signaling

The Notch family of transmembrane receptors plays a critical role in determination of cell fate during development, as well as in the maintenance of the somatic stem cell population (83). Within the family, there are four Notch receptors (*Notch1-4*) and five ligands (*Delta-like-1, 3, 4* and *Jagged-1, 2*) (84). Unlike normal receptor-ligand interactions the Notch ligands are not in a soluble form, but instead are present on neighboring cells as single transmembrane proteins (85).

Notch signaling begins with the binding of the Notch receptor to one of its ligands. Following binding, the extracellular domain of Notch is dissociated from the transmembrane domain and trans-endocytosed by the cell expressing the ligand. Upon removal of the extracellular domain,

Figure 3. Notch signaling pathway. Notch signaling occurs between two neighboring cells where one expresses the Notch receptor, while the other expresses one of the Notch ligands, Delta or Jagged. Binding of the Notch receptor leads to a proteolytic cleavage by the ADAM family of metalloproteases and γ -secretase, releasing the notch intracellular domain (NICD). Free NICD then translocates to the nucleus where it can bind to the promoter of Notch effector genes such as Hes1 and Hey1. The Notch downstream effectors then go on to effect cellular differentiation and stemness.

there are two proteolytic cleavages of the transmembrane domain, 1) by *ADAM10* or *ADAM17* and 2) by γ -secretase. These two cleavage events release the Notch intracellular domain (NICD) liberating it to translocate to the nucleus and act to alter transcription (84) (**Figure 3**). There are a wide variety of transcriptional targets of the NICD, including those pertaining to differentiation and stem cell maintenance (86). Interestingly, it has also been shown that increased Notch signaling correlates with increased activity of ALDH, as well as an observation that *CD133*⁺ cells have enhanced Notch signaling (87, 88). The overlap of numerous stem cell promoting pathways observed within the mentioned populations provides evidence that the CSC population may rely on multiple mechanisms to sustain the population.

In addition to functioning as a possible regulator for ALDH⁺ and *CD133*⁺ cells, Notch signaling has been demonstrated to impart a CSC phenotype in cells on its own. In NSCLC, increased Notch signaling has been shown to enhance the CSC phenotype, and correlates with poor patient prognosis in comparison to patients with low levels of Notch signaling (89, 90). It has been observed that increases in Notch signaling increase tumorigenicity, chemotherapeutic resistance, and radiation resistance (89, 91). The combination of these qualities demonstrate that cells with increased Notch signaling are more resistant to therapies than populations with reduced Notch activity and therefore limit the efficacy of therapeutic intervention, leading to decreased survival in clinic (92, 93).

Attempts to lessen the levels of Notch signaling have been attempted in NSCLC, utilizing γ -secretase inhibitors (94). This approach hopes to lessen the numbers of CSC within the cancer population, and thereby increase the ability of chemotherapeutics and radiation to eradicate the non-CSC population and the overall tumor. Currently the use of Notch inhibitors in the clinic has made it as far as Phase I clinical trial, and inevitably more research will be done in order to advance the efficacy and safety of these drugs in the future years (85).

Wnt Signaling

The Wnt pathway has long been known for its role in the regulation and maintenance of normal stem cells, such as those found in the gut epithelium (95). It has also been hypothesized that Wnt signaling can play a role in CSC regulation, where over-activation of this pathway can lead to the emergence of the CSC phenotype (96). Wnt signaling is overviewed well by He *et al*, and will be briefly reviewed here (97). First, Wnt signaling functions through at least three known pathways within cells (98). The first, and best known, is the canonical pathway. Canonically, Wnt ligands bind to either the Frizzled or low-density lipoprotein receptor-related receptors present on the extracellular surface. This binding then activates Disheveled, a protein responsible for the inactivation of glycogen synthase kinase (*GSK*). Inhibition of *GSK* inhibits the phosphorylation of β -catenin, thereby stabilizing it. Stabilization of β -catenin allows for its translocation to the nucleus where it is able to affect gene transcription (**Figure 4**).

Other, non-canonical Wnt signaling includes the planar cell polarity and Wnt/ Ca^{2+} pathways (98, 99). In addition the planar cell polarity pathway has also been demonstrated to lead to the activation of the small GTPases *Rho*, *Rac*, and *Cdc42* (100-102). Interestingly, the *Rac* family of GTPases has also been implicated in the maintenance of the CSC population (79).

While the canonical Wnt/ β -catenin pathway has been shown to play a crucial role in the maintenance of CSCs in malignancies such as colon, liver, breast, and leukemia, its role in NSCLS has yet to be clarified (103-106). Teng and colleagues were able to demonstrate that the canonical Wnt pathway was responsible for the maintenance of the CSC phenotype within A549 cells by upregulation of *OCT-4* and increasing levels of cyclin D1. It was discovered that this effect was caused by a decrease in the degradation of the basal level of β -catenin, hinting that the mechanism of action may effect the phosphorylation of free β -catenin (96).

Figure 4. Wnt Signaling Pathway.

The Wnt signaling pathway has been demonstrated to be important to the CSC population in a number of cancer types as well as in normal development. Under inactive conditions the Wnt effector protein, β -catenin (β -Cat), is sequestered by the destruction complex and phosphorylated, thus targeting it for ubiquitination and proteosomal degradation. The destruction complex is composed of Axin, glycogen synthase kinase 3 (GSK3), adenomatosis polyposis coli (APC) and casein kinase 1 (CK1). During active signaling, Wnt ligand binds to the LRP and Frizzled (Fz) receptors. Receptor activation causes the destruction complex to be recruited to the cytoplasmic tail of LRP by now phosphorylated Axin. Following relocation, Disheveled (DVL) is activated, thus inhibiting the action of GSK3. Loss of GSK3 activity leads to stabilization and accumulation of β -Cat within the cytosol. Accumulated β -Cat then translocates to the nucleus where it induces the transcription of Wnt target genes.

The normal functions of the Wnt pathway and its downstream effectors are known to support and regulate the CSC population within tumors (107). Wnt signaling plays an important role in certain cancer types, but is still understudied as a potential mechanism of CSC maintenance in NSCLC. Future research will need to be conducted in order to confirm the utility of the Wnt/ β -catenin pathway as a marker for the CSC population within NSCLC.

Summary of CD Markers and Signaling Pathways

Overall, the results from studies investigating CSC markers and pathways tend to contradict one another in NSCLC. One study may find that a population positive for a certain CD marker will display a CSC phenotype while the population without the CD marker is still capable of demonstrating CSC characteristics in subsequent studies. When investigation switches from CD markers to known somatic stem cell signaling pathways, the results become clearer, however investigators often focus on upstream regulators of known downstream stem cell promoting targets. An example of this would

be Wnt signaling resulting in increased expression of the somatic stem cell marker *Oct-4* (96). These results cause us to wonder at which level of regulation is the effect being perpetuated. Inhibiting upstream regulators of these stem cell pathways can have numerous effects on all their downstream targets, therefore in order to create safe and effective therapies, it is important to understand and target the effector molecules that often lie further downstream.

Attempts to define the CSC population and target it have provided researchers new angles by which to attack cancer, however it is not CSC markers and pathways that have made up the bulk of drugs designed to target cancer. It is instead a myriad of pathways and proteins that are overexpressed or overactive in malignancies. The belief is that the reduction or inactivation of these pathways will result in decreased tumor aggressiveness and better patient outcomes. Common oncogenic drivers such as *EGFR*, *K-Ras*, and *EML-ALK4* have all been shown to promote cancer initiation and progression, as well as become major foci of targeted therapies (108-110). Targeted therapy approaches often work well for a brief period of time, but over the course of a number of months these tumors become refractory to these therapies and the next line of therapy must be implemented. A great amount of research has been dedicated to understanding why cancer becomes resistant to these approaches and aims to design better drug formulations that may act in a more permanent fashion (111).

There also exists an interest in lesser-known, over-activated or over-expressed, proteins observed in cancers. These can include proteins that are important to certain cellular processes such as metabolism, cell motility, oxidative stress regulation, or autophagy (112-115). One of these proteins, NAD(P)H:quinone oxidoreductase 1 (*NQO1*), has garnered a lot of attention recently as it is observed to be greatly over-expressed in a number of cancer types, including NSCLC, but expressed at very low levels in normal tissues (116-118). This provides for an intriguing drug target as it can be a cancer directed therapeutic thus reducing unwanted off target effects. The role of *NQO1* in cancer, and cancer therapy is discussed below.

NAD(P)H:quinone oxidoreductase 1

Overview

NAD(P)H:quinone oxidoreductase 1 (*NQO1*) is a phase II detoxifying that takes part in the obligate two electron reduction of harmful quinones, utilizing either NADH or NAD(P)H as a cofactor. The quinones present within the cell can arise from exogenous or endogenous sources including, estrogen derived quinones, dopamine derivatives, and antitumor quinones (119-122). Unlike cytochrome p450 enzymes, this reduction results in a stable hydroquinone that does not produce harmful reactive oxygen species due to the presence of a free electron (123, 124). The stable hydroquinone formed by *NQO1* is later conjugated to glutathione and excreted from the cell. Interestingly, *NQO1* has been demonstrated to be greatly overexpressed in a number of tumor types in comparison to normal tissues (118, 125-127). These observations present researchers with the possibility of being able to specifically target tumor cells utilizing *NQO1* as a drug target, thus sparing normal cells due to an inherent low expression of *NQO1*.

NQO1-specific antitumor approaches are being investigated utilizing the ortho-naphthoquinone, β -lapachone (β -lap). Isolated from the lapacho tree, this compound has been used medicinally for centuries and has recently garnered respect in cancer therapy (128). β -lap's mechanism of action is strictly dependent on the expression of *NQO1*. *NQO1* catalyzes the formation of an unstable hydroquinone form of β -lap that spontaneously reverts back to the parent compound, thus resulting in the release of reactive oxygen species. The level of reactive oxygen species released is extremely high resulting in DNA damage, *PARP1* hyper-activation, nucleotide depletion, and eventual cell death (129). Promising pre-clinical results have supported β -lap's promotion to clinical trial. Known clinically as ARQ-501, or ARQ-761, β -lap has reached stage II clinical trials in prostate cancer, as well as a currently enrolling stage I trial for other solid malignancies (NCT01502800). In addition to stand-alone therapy with these compounds, research is also being conducted to investigate the synergistic effects of β -lap with radiation (130). It has been observed that ionizing radiation induces expression of *NQO1* leading to a synergistic effect when β -lap is administered following radiation (131, 132). Despite the promise observed with β -lap treatment, caution must be taken when attempting to treat patients with *NQO1* directed therapy due to the presence of polymorphisms.

Polymorphisms

Wild-type *NQO1* protein is targetable via β -lap treatment, however two polymorphisms of *NQO1* exist. The *2 polymorphism, a cysteine to threonine substitution at nucleotide position 609, results in a proline to serine substitution at position 187 in the amino acid sequence (133, 134). The result of this polymorphism is a highly unstable protein that has undetectable activity and is quickly degraded (135). This form of the polymorphism is predicted to occur in 5-20% of patients (136). Surprisingly, the presence of this polymorphism has been linked to the susceptibility of a number of diseases including cancer, as well as often predicting worse outcomes (137-143). These results counter what is observed in other reports where wild-type *NQO1* expression leads to a worse overall survival, indicating a pro-tumorigenic role for *NQO1* (117, 144-146). The *3 polymorphism exists due to a cysteine to threonine substitution at nucleotide position 465 that results in an arginine to tryptophan substitution at amino acid position 139 (147). This polymorphism results in an alternative splicing event that renders substantially less protein in cells harboring the polymorphism (148). Studies investigating the prevalence of these polymorphisms demonstrate that the *3 polymorphism occurs at a much lower rate (across different ethnic groups) than the *2 mutation (149).

Regulation

Expression of the *NQO1* gene is controlled by a number of factors including antiestrogens, electrophile response elements, and antioxidant response elements (ARE) (150-152). The most commonly discussed is its regulation by transcription factors binding to the ARE in the promoter region. The ARE consensus sequence, TMA_nRTGAYnnnGCR_{www}, is capable of binding a number of bZIP transcription

factors including *Nrf1*, *Nrf2*, *Maf*, *Jun*, *Fos*, and *Raf* (153). Additionally, it has been seen that *ERK* signaling can result in the increased expression of ARE regulated genes (154).

The canonical regulation of *NQO1* is provided by the transcription factor NF-E2-related factor 2 (*Nrf2*) (153). Much like *NQO1*, *Nrf2* is also a commonly studied protein in cancers as it too has been observed to have an increased expression in a number of cancer types (155, 156). *Nrf2* exists in equilibrium with its negative regulator Kelch-like ECH-associated protein 1 (*Keap1*). *Keap1* binds to *Nrf2* and sequesters it in the cytoplasm, restricting the transcription factor

Figure 5. *Nrf2* regulates the expression of *NQO1* and other oxidative stress regulating genes. The expression of *NQO1* is under the control of the *Nrf2/Keap1* pathway. Under homeostatic conditions, *Nrf2* is bound by *Keap1* in a complex that includes the Cul3 ubiquitin ligase. Under these steady state conditions Cul3 is capable of polyubiquitinating (grey circles) *Nrf2*, thus targeting it for proteosomal degradation. Under conditions of oxidative stress, *Keap1* acts as a sensor that then releases *Nrf2*. The free *Nrf2* is then capable of translocating to the nucleus and binding to antioxidant response elements (ARE), along with other co-activators (*Maf* pictured) of oxidative stress regulating genes, including *NQO1*.

from interacting with the DNA and inducing transcription. The binding of *Keap1* to *Nrf2* allows for the Cullin 3 (Cul3)-based E3 ubiquitin ligase to associate with the complex, inducing the poly-ubiquitination of *Nrf2* and subsequent proteosomal degradation (157). Under periods of oxidative stress, the *Nrf2/Keap1* association is lost due to the oxidation of cysteine residues on *Keap1* (158). Loss of association allows for *Nrf2* accumulation and relocation to the nucleus. Upon localization to the nucleus, *Nrf2* can bind to the AREs of its canonical targets and induce their expression (159). In addition, roughly 15% of lung cancer patients harbor *Keap1* mutations that inhibit the interaction of *Keap1* with *Nrf2* that leads to increased activity of *Nrf2* (156) (**Figure 5**).

Interestingly, *Nrf2* has recently been demonstrated to regulate the expression of Notch and control the division and proliferation of normal bronchial stem cells (160). The report directly connects oxidative stress regulation to the maintenance of stem cell populations, and suggests that this may also be the case in the cancer stem cell population. Of note, it has also been demonstrated that increased Notch activity results in an increase in *Nrf2* expression in liver cancer (161). These observations suggest that there is a reciprocal regulation of these two proteins by one another (162).

Summary

Research into the role that *NQO1* plays in promotion of tumorigenesis and why it tends to be overexpressed in a number of malignancies is essentially non-existent. The majority of research focus on tumor promotion is focused on the canonical *NQO1* regulator, *Nrf2* and its tumor supportive role (163, 164). Given that there is little work focused on understanding the role of *NQO1* in tumorigenesis, it comes as no surprise

that the importance of this oxidative stress regulator, in the CSC population, has also not been evaluated. Here we present work that demonstrates *NQO1* plays a vital role in NSCLC initiation and progression, as well as being imperative in the maintenance of the CSC population in NSCLC. These results provide novel insight into the greater involvement of *NQO1* in the promotion and progression of NSCLC, beyond its simple overexpression and usefulness as a drug target.

Chapter 2

Depleting tumor-*NQO1* Potentiates Anoikis and Inhibits Growth of NSCLC

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Running title: *Depleting tumor-NQO1 levels inhibits NSCLC growth*

Abstract

The fundamental role that NAD(P)H/quinone oxidoreductase 1 (*NQO1*) plays, in normal cells, as a cyto-protective enzyme guarding against stress induced by reactive oxygen species (ROS) is well documented. However, what is not known is whether the observed overexpression of *NQO1* in neoplastic cells contributes to their survival. The current study discovered that depleting *NQO1* expression in A549 and H292 lung adenocarcinoma cells caused an increase in ROS formation, inhibited anchorage-independent growth, increased anoikis sensitization and decreased 3-D tumor-spheroid invasion. These *in vivo* data further implicate tumor-*NQO1* expression in a pro-tumor survival role, since its depletion suppressed cell proliferation and decreased lung tumor xenograft growth. Finally, these data reveal an exploitable link between tumor-*NQO1* expression and the survival of lung tumors since *NQO1* depletion significantly decreased the percentage of ALDH^(high) cancer cells within the tumor population.

Introduction

Lung cancer is the leading cause of cancer related deaths in the U.S. (2). Over the past decade some improvement has been made toward the goal of increasing overall survival in lung cancer patients. These improvements have mostly been due to technological advances allowing early diagnosis of lung cancer as well as improved molecular based therapeutic approaches (165). However, with 5-year survival rates at 15% or less, novel mechanism based therapeutic approaches are still desperately needed.

NADPH quinone oxidoreductase-1 (*NQO1*) is an inducible two-electron oxidoreductase that is highly overexpressed in many solid tumors including breast, pancreas and non-small cell lung cancer (NSCLC) (116, 118, 166-171). *NQO1* is an essential phase II detoxification gene and as such plays a critical role in both detoxification and bio-activation of many DNA damaging quinones (167). As a chemopreventive gene, *NQO1* has been shown to detoxify a broad spectrum of quinone substrates and it plays a role in reactive oxygen species (ROS) scavenging by generating antioxidant forms of alpha tocopherol (167, 172).

In our past investigations we demonstrated that *NQO1* bioactivated several anticancer quinones including b-lapachone (173) and more recently deoxynyboquinone (DNQ) (174). Our previous *in vitro* studies determined that *NQO1* is a viable target for developing personalized lung cancer therapy since tumor-*NQO1* levels are often 5-20 fold greater in lung tumors as compared to the levels of *NQO1* observed in associated normal tissues (170). Thus, targeting *NQO1* with anticancer quinones has become a feasible option for preclinical anticancer studies. Furthermore, our *in vivo* studies with anticancer quinones and novel drug delivery formulations, has led to a surge in interest in *NQO1*-bioactivated anticancer quinones (174, 175), resulting in clinical trials for treatment of various solid tumors. However, there is still very little known as to why *NQO1* levels are so vastly overexpressed in solid tumors. More specifically, no studies have addressed whether reducing tumor-*NQO1* levels affects processes critical to tumor survival and proliferation, including anchorage-independent growth, escape from apoptosis and the ability to invade and metastasize.

In the current study we hypothesized that depleting *NQO1* expression levels in NSCLC tumors would have deleterious effects on cell proliferation and survival. Our rationale for this hypothesis stemmed from numerous reports suggesting that cancer cells must regulate oxidative stress levels to prevent death from toxic levels of ROS created in their microenvironment as part of a host defense response (176). Thus, one strategy to protect tumor cells from lethal levels of ROS stress is to activate, or hijack, pathways that regulate the expression levels of antioxidant genes. Importantly, a primary regulator of oxidative stress is the transcription factor *Nrf2* whose role is to activate antioxidant gene expression; and its own overexpression has been associated with enhanced tumorigenesis (163, 177, 178). One of the many transcriptionally activated antioxidant genes regulated by *Nrf2* is *NQO1*, and numerous studies have shown that *NQO1* levels in various tumors are elevated in comparison to associated

normal tissues (116, 118, 170). Here we show that depletion of *NQO1* expression levels, in various NSCLC cell lines, decreased the tumor cells ability to form colonies in anchorage-independent growth assays. The inability of *NQO1*-depleted NSCLC cells to form tumor colonies in anchorage-independent assays correlated with increased reactive oxygen species formation, an increase in anoikis sensitization and a decrease in cell proliferation rates. Our data also show that depletion of *NQO1* expression levels inhibited the ability of NSCLC cells to invade in 3D-tumor spheroid assays. Our *in vivo* data show that loss of tumor-*NQO1* expression in NSCLC cells inhibited tumor growth as compared to controls. Finally, we show that *NQO1* knockdown decreases the percentage of ALDH^(high) cancer cells, suggesting that the depletion of *NQO1* decreases tumorigenicity by eliminating the cancer stem cell population within the tumor. Together these novel findings illuminate the role of *NQO1* in tumors, and suggest that depleting tumor-*NQO1* levels disrupts the protective barrier against ROS provided to cancer cells by elevated tumor-*NQO1* expression levels. Thus, *NQO1* depleted tumor cells are more susceptible to oxidative stress and their overall growth and survival is inhibited due to increased cell death, and reduced proliferation of the cancer stem cell population.

Materials and Methods

Reagents

NQO1 activity assay kit (Abcam), Cell death detection ELISA kit (Roche Applied Sciences), Seaplaque agarose, SeaKem agarose, 1N Sodium Hydroxide and Rat tail collagen type I (Fisher Scientific), Noble agar (Becton, Dickinson), 10X DPBS (Hyclone), Cyquant cell proliferation assay kit and 2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester, DCFDA (Lifetechnologies). The *NQO1* inhibitor Mac220 was a generous gift from Dr. David Ross, University of Colorado Anschutz Medical Center.

Cell growth and maintenance assays

H292, HCC1171 and non-transformed, non-tumorigenic human bronchial epithelial (HBEC) cell lines were a generous gift from the laboratory of Dr. John D. Minna, UTSW Medical Center at Dallas. A549 and H596 cells were previously described (170). A549, H596, H292 and HCC1171 cell lines were cultured in DMEM (Lonza) containing 10% fetal bovine serum (FBS) and 1% L-glutamine. HBEC cells were cultured in Keratinocyte Serum-Free Media with supplements (Invitrogen). All cell lines were incubated at 37°C at 10% CO₂.

Western Blotting

Protein lysates were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with 5% milk in PBST for 1 hour at room temperature, and then incubated overnight with β -actin (1:5000 in 3% BSA, Santa Cruz Biotechnology) at 4°C. Blots were washed in PBST and incubated for 1 hour with 1:5000 dilution of goat-antimouse IgG-HRP in 5% milk in PBST. The process was repeated using a 1:5000 dilution of monoclonal *NQO1* antibody (clone A-180, Santa Cruz Biotechnology). Pierce ECL western blotting substrate (Thermo Scientific) was used to visual bands on Hyblot-CL autoradiography film (Denville Scientific). For *PARP-1* cleavage assays, A549 (shCtr-R and shNQO1) tumors were harvested post

mortem and sonicated in PARP-lysis buffer as described previously (173, 179). Extracts were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. *PARP-1* protein was visualized using a monoclonal *PARP-1* antibody (Santa-Cruz, clone F-2) at a 1:1000 dilution.

Patient survival analysis

Three separate survival analyses were performed on overall survival data from NSCLC patients obtained from 3 TCGA data sets (<https://tcga-data.nci.nih.gov/tcga/>) (180-182). Gene expression values used were reported from Affymetrix U133A Microarray data. Patient data with valid gene expression levels were used to estimate medians and bounds for upper and lower quartiles. Patients were categorized into two groups based on whether the values of gene expression were above the upper quartile bound and below the lower quartile bound. Kaplan–Meier survival graphs were plotted, and log-rank tests were performed using GraphPad Prism.

Transient and stable *NQO1* protein knockdown assays

The human shRNA-*NQO1* retroviral vector (RHS1764-9691437) was purchased from Open Biosystems. A stable shRNA knockdown cell line (A549-shNQO1) and vector control (A549-shCtr-R) was generated by infecting A549 cells with polybrene-supplemented medium obtained from Phoenix packaging cells transfected with the human retrovirus vector targeting *NQO1* or non-silencing control vector as described previously (168). Human shRNA-*NQO1* lentiviral particles (sc-37139) and controls were purchased from Santa Cruz Biotechnology. Stable shNQO1 lentiviral knockdown (A549-shNQO1-B) and control (A549-shCtr-L) lines were generated by infecting A549 cells with polybrene-containing culture medium (5 µg/mL) and 10 µL of the lentiviral particles were added directly to the culture medium. Medium was changed 24 hours after transfection. After 48 hours shNQO1 containing cells were isolated by limited dilution in media containing puromycin (2 µg/mL) and screened for *NQO1* expression levels by Western blot. Similar experiments were performed with H292 cell lines to create H292-shNQO1-B and H292-shCtr-L cell lines. H596 cells, which are *NQO1* null, were infected with retrovirus particles from a retroviral control (LPC-X) or retroviral *NQO1* (LPC-*NQO1*) vector as described previously (9). For transient *NQO1* knockdown, siRNA-*NQO1* or scramble control siRNA (Santa Cruz Biotechnology) was transiently transfected into HCC1171 or H596 cell lines (Lipofectamine 2000, Life Technologies) using the Life Technologies protocol. Cells were harvested after 48 hours and analyzed for *NQO1* protein expression or enumerated using a hemocytometer for use in invasion assays.

***NQO1* activity assays**

To analyze endogenous *NQO1* levels we used an *NQO1* activity assay kit (Abcam). Briefly, cell pellets, containing 2×10^7 cells, were collected for each cell line. Pellets were solubilized in 1X extraction buffer on ice for 20 minutes. Samples were then centrifuged at $18,000 \times g$ for 20 minutes at 4°C. Supernatants were transferred to new eppendorf tubes and aliquots were stored at -80°C. Protein concentration was determined using the Bio-Rad protein assay method. Samples were diluted to 2X the working concentration of 5 µg/mL with supplemented buffer. Two wells were prepared for each

sample (one well with and one well without inhibitor). 50 μ L of each cell line was plated in triplicate in 96 well plates provided with the kit. The reaction buffer and the reaction buffer plus inhibitor were prepared according to the manufacturer's calculation table. The reaction buffer plus inhibitor were added to the samples first. Reaction buffer without the inhibitor were added last. Absorbance was measured at 440 nm every 20 seconds for 5 minutes using the Synergy-H1 Hybrid microplate reader. The plates were shaken before and after each reading.

In vitro survival assays

Long-term survival assays based on DNA content after 7-10 days of growth were conducted in 48 well dishes as previously described (170, 173). Cells were treated with vary doses of ARQ-761 (aka b-Lapachone) in the presence or absence of the *NQO1* inhibitors dicoumarol or 5 μ M Mac 220.

Cell Proliferation Assays

To determine cell proliferation rates we used the CyQUANT cell proliferation assay kit (Life Technologies) and followed the manufacturers protocol. Briefly, standard curves were generated by pelleting 1×10^6 cells for all cell lines. Pellets were resuspended in 1 mL of CyQUANT GR/cell-lysis buffer and vortexed briefly. A dilution series in one row of a 96 well microplate ranging from 50 to 50,000 cells per cell line in total volumes of 200 μ L were plated along with a 200 μ L control well with no cells and incubated for 5 minutes in the dark at room temperature. Using a Synergy-H1 Hybrid Reader (Bio Tek), fluorescence was measured at excitation 480nm and at 520nm emission. For proliferation, cells were plated out in 6 wells at 5,000 cells per well in a total of 200 μ L of a 96 well plate. Multiple plates were seeded using the same starting concentration and cultured at 37°C and 10% CO₂ until desired time to collect the plates. Plates were collected at 0, 24, 48, and 72 hours by inverting the plates and blotting on a paper towel to remove medium from the wells. Plates were stored at -80°C until all plates were collected. Plates were thawed at room temperature and 200 μ L of CyQUANT GR/cell-lysis buffer was added. Plates were incubated for 5 minutes in the dark at room temperature.

Anchorage-independent growth assays

For A549 cell lines, a 1.5% SeaPlaque Agarose (SPA) mixture was made by slowly adding SPA to PBS and autoclaving. 0.5% SPA was created by diluting the 1.5% stock SPA 1:3 with culture media. 1 mL of the 0.5% SPA mixture was added to each well of a 6 well plate to create a bottom layer and allowed to solidify at room temperature for 15-20 minutes. Cells were counted and suspended at 750 cells/mL in a separate 0.5% SPA mixture. 2 mL were added to each well on top of the bottom layer and allowed to solidify for 30-45 minutes at room temperature to create a cell layer. A 0.3% SPA mixture was created by diluting the 1.5 % stock SPA 1:5 with culture media. 1 mL of the 0.3% SPA mixture was added to each cell layer and allowed to solidify for 20-30 minutes at room temperature to create a top layer. 250-500 μ L of culture media was added onto the top layer to prevent from drying out. A similar method was used where SeaKem Agarose was substituted for SeaPlaque Agarose yielding similar results. Plates were wrapped in parafilm and placed at 37°C. 250-500 μ L of new culture media was added every week.

Plates were imaged after 3 weeks (A549 cells) and 6 weeks (H292 cells) using an Epson V700 photo scanner. The enumeration of colonies present in each dish was quantified using imageJ software.

Cell Death Elisa (CDE, anoikis) assays

A cell death detection ELISA kit was used to determine the level of detachment induced cell death (anoikis). Briefly, cells were seeded at a density of 250,000 cells in a 10cm dish for 48 hours prior to plating for assay. 2.0 mL of 0.5% methylcellulose and culture medium mixture was added to poly-HEMA coated plates and allowed to equilibrate in the incubator for 1 hour at 37°C. 150,000 cells were seeded per well in the 0.5% methylcellulose mixture and incubated at 37°C for the 24 and 48 hour time points. For the zero hour time point cells were placed directly into Eppendorf tubes and placed on ice. Cells were lysed with 100 µL of CDE lysis buffer at 4°C for 20 minutes. Cells were pelleted for 12 minutes at 4°C at 13,000 rpm. 75 µL of the supernatant was transferred to a new tube and stored at -80°C until all time points were collected. All samples were processed using the cell death detection ELISA kit with the manufacturer's protocol. Absorbance values were recorded using the Synergy-H1 Hybrid reader (Bio Tek) at 405 nm. Values were calculated by subtracting the zero hour time point from the 24 and 48 hours time points.

Invasion assays

Invasion assays were performed as described previously (183). Briefly a 1.5% noble agar/ PBS mixture was made and then autoclaved. Using a multi-channel pipette, 100 µL of the noble agar mixture was added to each well of a 96 well plate. Cells were counted and suspended at a density of 50,000 cells/mL. 200 µL of this suspension was added to the 96 well plates once the noble agar was solidified. Plates were allowed to sit for 1-3 days depending on cell line until spheroids were formed. Once spheroids were formed, a 400 µL base layer mixture of 10X DPBS, 1N sodium hydroxide, sterile water and rat tail collagen was added to a 24 well plate and allowed to solidify at 37°C for 30 minutes. Using a nucleofector pipette, spheroids were added one at a time to Eppendorf tubes. 3 spheroids were added to each tube. Spheroids were centrifuged at 1,000 rpm for 3 minutes. The media was removed from each tube using a nucleofector pipette. A collagen cell layer mixture was made and 500 µL were carefully added to each tube, one at a time, and spheroids were added to the 24 well plates. The cell layer was incubated at 37°C for 1 hour. 1 mL of culture media was added to the top of the cell layer to prevent the collagen from drying out. Once media was added, zero hour images were taken at 5X (A549 and HCC1171 cells) or 10X (H292) magnification using an inverted microscope (Axiovert 200M, Zeiss). Images were taken every 24 hours until invasion distance was outside of the focal field of view. The scale bar in each image represents 100 microns. Using the AxioVision software, the invasion distance was calculated by subtracting the initial spheroid radius from the invasive distance at the final time point.

DCFDA staining for ROS studies

500,000 cells were plated in 10 cm dishes containing 7 mL of 0.5% Methylcellulose mixture. Cells were incubated for 24 hours at 37°C and 10% CO₂. After 24 hours pellets

were collect by centrifuging at 1,000 rpm for 2 minutes. Pellets were washed 1X with PBS and centrifuged at 1,000 rpm for 2 minutes. Pellets were resuspended in DMEM/F-12 without phenol red, which contained either 5 μ M DCFDA (stained) or DMSO (unstained control), and transferred to flow cytometry tubes. Cells were incubated at 37°C and 10% CO₂ for 30 minutes. Cells were then pelleted again at 1,000 rpm for 2 minutes. Pellets were resuspended in 500 μ L of DMEM/F-12 without phenol red and placed at 37°C and 10% CO₂ for 15 minutes to equilibrate and then samples were read using the LSR Fortessa (BD Biosciences).

In vivo tumorigenesis and survival assays

For tumorigenesis assays 4-6 week old female athymic nude mice (Charles River) were subcutaneously injected on the right flank with A549 shCtr-R and A549 shNQO1 cells on the left flank. Mice were stratified into 3 groups containing initial tumor injection concentrations of 5 million, 2.5 million or 1 million cells of each cell type. 10 mice were used for each group. Tumor growth rates were monitored by caliper measurements using the formula ($L \times W^2/2$). Statistical significance between A549-shNQO1 and A549-shCtr-R tumor growth rates, at each concentration, was calculated using an unpaired, two tailed Students t-test.

In separate studies, to compare the effect that *NQO1* depletion had on overall survival, 4-6 week old athymic female nude mice (Charles River) were subcutaneously injected with A549-shCtr-R or A549-shNQO1 cells into their flanks. Tumor growth rates were monitored by caliper measurements using the formula ($L \times W^2/2$). Tumor growth was assessed until the tumors reached the set volume of 1000 mm³. Post mortem, tumors were collected for evaluation of *NQO1* expression. Log-rank test were applied to survival analyses (Kaplan-Meir). All statistical significance assessments were conducted using Graph-Pad Prism 6 software.

All animal studies were performed in accordance with the animal care policies of West Virginia University and were approved by the West Virginia Animal Care and Use Committee.

Aldefluor activity assays

The percentage of cells that were ALDH^(high) within the various cell populations assayed was determined using the Aldefluor Kit (Stemcell Technologies). The kit was used according to the manufactures protocol. Briefly, two flow cytometry tubes, per cell line, were labeled as control or test. Cells were trypsinized and pelleted at 1,000 rpm for 1-2 minutes. 1×10^6 cells were then counted out for both shCtr-R and shNQO1 cell lines. Pellets were washed 1X with PBS and resuspended in 1mL of the Aldefluor Assay Buffer provided and transferred to the “test” tube. 5 μ L of the DEAB (ALDH inhibitor) was added to the “control” tube and was immediately recapped. 5 μ L of the Aldefluor reagent was added to the “test” tube and was vortexed immediately. After the “test” tube had been vortexed, 500 μ L of the Aldefluor Assay Buffer was transferred from the “test” tube to the “control” tube and the “control” tube was immediately vortexed. That procedure was repeated for each cell line. Once all cells line were stained, the “test” and “control” tubes were incubated at 37°C for 30 minutes. After the incubation period, tubes were

centrifuged at 250 rpm for 5 minutes. Supernatant was removed and pellets were resuspended in 500 μ L of Aldefluor Assay Buffer and samples were placed on ice. A549 samples were assessed using a Fortessa flow cytometer and Mia PaCa and PC3 cell lines were analyzed using a FACS Calibur flow cytometer (Becton Dickinson). Final data analysis was performed using FCS Express software.

Cell viability assays

HBECs were transiently transfected with siRNA-*NQO1* or scramble control according to the protocol described above. After the 48 h transfection period, cells were enumerated and seeded at a density of 20,000 cells/ well in 96 well plates (white). The following day, the viability of cells in each group (8 wells/ group) was assessed by adding 100 μ L of CellTiter-Glo (promega) to each well. Luminescence was detected using a Synergy-H1 Hybrid reader (Bio Tek).

Statistical analysis

Statistical differences were determined by using Student's *t* tests, and *p* values were reported. All statistical analyses were performed using Graph-Pad Prism 6 software, and considered significant when *p* values were < 0.05.

Results

Elevated *NQO1* expression predicts poor survival in NSCLC patients.

In previous studies our laboratory, as well as other investigators, reported that *NQO1* expression levels were highly elevated in lung cancer patient tumor versus associated normal lung tissue (170, 184). Elevated tumor-*NQO1* levels have provided a distinct advantage for developing *NQO1*-directed anticancer therapeutics such as b-lapachone and deoxyniboquinone (174, 175). However, in more recent retrospective investigations of patient outcomes, a strong correlation between elevated tumor-*NQO1* expression levels and poor patient survival in various cancer types including breast and ovarian cancers has emerged (185, 186). Thus, we sought to determine whether elevated *NQO1* expression in lung tumors also confers a survival disadvantage in lung

cancer patients. We analyzed gene expression and survival data from NSCLC (lung adeno- and squamous cell carcinoma) patients within The Cancer Genome Atlas (TCGA) (180-182). Patients were stratified into high and low *NQO1* expressers based on a quartile bound cutoff. With this cutoff, a total of 191 patients were identified as high expressers and 244 patients were identified as low expressers. Our data, in three separate analyses, show that lung cancer patients with high tumor-*NQO1* expression levels have worse overall survival (**Figure 1A and Supplemental Figure 1**). Our data are consistent with the aforementioned reports of poor overall survival in breast and ovarian cancer patients whose tumors had high *NQO1* expression levels (185, 186). These data, as well as our laboratory observations that *NQO1* levels increase during the process of transformation, suggested that cancer cells increase *NQO1* expression levels as part of a pro-survival strategy during tumorigenesis, and that depleting *NQO1* levels could possibly eliminate this survival advantage.

Depleting tumor-*NQO1* levels inhibits anchorage-independent growth and invasion of NSCLC.

To investigate whether depleting *NQO1* would alter the growth of lung cancer cells we used *NQO1* shRNAs to establish stable knockdown of *NQO1* in A549 and H292 NSCLC cell lines. Our data show that sh*NQO1* knockdown in A549 cell lines using a retroviral vector (sh*NQO1*) or lentiviral vector (sh*NQO1*-B) caused a significant decrease in *NQO1* protein expression levels (**Figure 1B and Supplemental Figure 2A**), which correlated with loss of *NQO1* activity (**Figure 1C**). Similar results are shown for *NQO1* knockdown in H292 cells (**Figures 1B lower panel and 1D**). The parental A549 cells have nearly 12 fold higher levels of *NQO1* activity as compared to the levels of *NQO1* activity detected in H292 cells. Thus, these two cell lines with their distinct differences in *NQO1* activity levels, also serve as an internal comparison to determine whether patients with lower *NQO1* levels in their tumors could also benefit from therapeutic strategies aimed at depleting *NQO1* expression.

A hallmark of oncogenic transformation is the newly acquired ability of a transformed cell to grow in an anchorage-independent environment. This acquired phenotype also increases the invasive and

Figure 2. Depleting *NQO1* expression levels inhibits growth of NSCLC cells in soft agar. In **A**, A549 shCtrl-R and A549 sh*NQO1* cells were analyzed for their ability to form colonies and grow in soft agar. Photomicrographs shown are representative of experiments performed in sextuplet. In **B-D**, graphical representation of enumerated colonies for A549 sh*NQO1*, A549 sh*NQO1*-B and H292 sh*NQO1*-B cells versus A549 shCtrl-R, A549 shCtrl-L and H292 shCtrl-L cells. In **B**, *p* values for A549 sh*NQO1* vs A549 shCtrl-R (*p* < 0.0001). In **C**, *p* values for A549 sh*NQO1*-B vs A549 shCtrl-L (*p* = 0.0041). In **D**, *p* values for H292 sh*NQO1*-B vs H292 shCtrl-L (*p* = 0.0114).

metastatic potential of transformed cells. In previous reports A549 and H292 cell lines served as metastatic models for *in vivo* studies (187, 188). Thus, we hypothesized that stable shRNA knockdown of *NQO1* in A549 and H292 cells would be sufficient to determine whether *NQO1* depletion affected tumor growth in anchorage-independent colony forming assays (also referred to as soft agar assays). In **Figures 2A-D and Supplemental Figure 3** our data show that stable depletion of *NQO1* significantly inhibits the growth of A549 and H292 cells in soft agar assays. Interestingly, the inhibition of *NQO1* expression in A549 cells using the shNQO1 vector was substantially greater than what was observed with the shNQO1-B vector. Thus, the higher *NQO1* activity observed in shNQO1-B A549 knockdown cells (**Figure 1 C**) correlated with more colony growth (**Figure 2C and Supplemental Figure 2B**). These data suggest that the degree of *NQO1* activity loss affects the ability of cells to grow in soft agar. In addition to *NQO1* shRNA knockdown studies we examined the effect of dicoumarol and Mac 220, *NQO1* inhibitors that mimic the co-factor NAD(P)H which is required for *NQO1* activity (189), on colony growth. Our data show that the *NQO1* inhibitors dicoumarol and Mac220 also significantly inhibited the growth of A549 cells in soft agar colony forming assays (**Supplemental Figures 4 and 5**), further confirming that anchorage-independent growth in these cells is affected by the loss of *NQO1* activity.

To further confirm the role of *NQO1* in anchorage-independent growth we examined the effect of *NQO1* overexpression in H596 cells, which are *NQO1* null due to the *2 polymorphism. Our data show that *NQO1* overexpression in H596 cells (H596-LPC-NQO1) caused significantly more colony growth as compared to vector only H596-LPCX cells (**Supplemental Figure 6**). In contrast, our data show that transient knock down of *NQO1* in H596-LPC-NQO1 cells results in significant loss in their ability to grow in soft agar (**Supplemental Figure 7**). These data further indicate that *NQO1* plays a role in the survival of cells in an anchorage-independent environment.

The ability to survive in an anchorage-independent environment is uniquely tethered to a cancer cells ability to invade and metastasize (190). Thus, we employed an *in vitro* 3- dimensional spheroid invasion assay (183, 191) to address the role that *NQO1* plays in the process of tumor cell invasion. Our data show that stable knock down of *NQO1* in A549 cells decreased the overall area of lung tumor spheroids (**Supplemental Figure 8A**) and inhibited the invasive progression of lung tumor spheroids in A549 and H292 cells (**Figures 3 A-D and Supplemental Figure 8B**). In addition to A549 and H292 cells, our data show that transient depletion of *NQO1* expression using siRNA also inhibited invasion in HCC1171 lung cancer cells (**Supplemental Figure 9**). In contrast to *NQO1* knockdown studies, when *NQO1* was overexpressed in H596 cells a significant increase in invasion was observed (**Supplemental Figure 10**). Together these data demonstrate that *NQO1* levels are critical for anchorage-independent growth and the invasion of lung cancer cells.

***NQO1* depletion elevates ROS levels and sensitizes cells to anoikis.**

Previous investigations have shown that *NQO1* can act as a scavenger of ROS (192), thus we hypothesized that depleting *NQO1* in our lung cancer models would increase endogenous levels of ROS. As expected, our data show that depletion of *NQO1* in A549 cells caused an increase in oxidative stress as indicated by the increased DCFDA staining (a general ROS indicator including H_2O_2 levels (**Figures 4 A-B**), supporting our hypothesis that endogenous ROS levels are increased in lung cancer cells when tumor-*NQO1* levels are depleted.

In transformed cells the intracellular production of ROS is tightly regulated to prevent programmed cell death (193). Excessive ROS production can lead to apoptotic catastrophe, and cells that escape apoptosis are resistant to detachment induced cell death, also known as anoikis (194). Anoikis resistant cells are capable of continued

proliferation and distant tumor formation (195). Thus far, our data show that depleting *NQO1* prevents anchorage-independent growth and increases ROS stress levels. Therefore, we further hypothesized that the inability of *NQO1* depleted cells to grow in an anchorage-independent environment was linked to anoikis sensitization caused by increased levels of ROS. To test this hypothesis we performed cell death ELISA assays on *NQO1* knockdown cell models. Our cell death assays show that loss of *NQO1* in A549 and H292 cells significantly increased sensitization to anoikis (**Figures 4C-D**). These data suggest that depletion of *NQO1* expression in lung cancer cells increases oxidative stress and potentiates detachment induced cell death.

Depleting tumor-*NQO1* expression levels decreases cell proliferation and *in vivo* tumor growth.

Uncontrolled proliferation is a hallmark of malignant neoplastic cells, thus novel approaches to reduce uncontrolled cell proliferation are of paramount importance in the development of anticancer strategies. Our current data show that loss of *NQO1* decreases tumor growth in soft agar and increases sensitization to anoikis. Thus, we hypothesized that depletion of tumor-*NQO1* levels would significantly decrease the ability of cells to proliferate. Our data show that A549 and H292 *NQO1* knockdown cells had significantly lower rates of proliferation as compared to their respective controls (**Figures 5 A-B**). These data suggest that depletion of *NQO1* inhibits cell growth by inducing apoptosis caused by detachment induced cell death.

To address the role that *NQO1* depletion plays *in vivo* tumor growth, varying concentrations (1, 2.5 and 5

million) of A549 shNQO1 and A549 shCtr-R cells were implanted subcutaneously into athymic mice and tumor growth and overall survival rates were evaluated. Our *in vivo* xenograft data clearly show that a significant growth disadvantage is observed in A549-shNQO1 cells at each concentration of cells implanted as compared to A549-shCtr-R cells (**Figures 5 C and E**). In addition to significantly reducing *in vivo* tumor growth rates, the depletion of tumor-NQO1 expression levels in animals bearing A549-xenografts increased their overall survival as compared to animals bearing A549-shCtr-R xenografts (**Figure 5 D**). We also observed that tumor-NQO1 levels remained depleted in A549-shNQO1 xenografts as illustrated by our *in vivo* western-blot analysis for NQO1 protein expression (**Figure 5 F**). Interestingly, a substantial difference in PARP-1 proteolysis was observed in A549-shNQO1 tumors as compared to A549-shCtr-R tumors, further supporting our anoikis data that suggest that loss of NQO1 leads to increased apoptosis (**Figure 5 F and Supplemental Figure 11**).

NQO1 depletion reduces the percentage ALDH^{high} cells in the tumor cell population.

We have shown that knockdown of NQO1 expression in lung cancer cells decreased clonogenic growth *in vitro* and tumor growth *in vivo*. Numerous studies have reported that cancer stem cell populations are responsible for increased tumorigenicity and resistance to therapeutics. Thus, we sought to determine if NQO1 affected this critical population of cells. Previous work has shown that one of the most reliable cancer stem cell markers is aldehyde dehydrogenase (ALDH) (196, 197). Although several isoforms of ALDH exists, a common assay used to define the ALDH^(high) stem cell population is the Aldefluor assay (196). In our studies we tested the hypothesis that NQO1 depletion caused less tumor growth due to depletion of ALDH^(high) cells. Our data clearly show that there is a significant decrease in the ALDH^(high) population in A549 shNQO1 cells as compared to A549 shCtr-R cells (**Figure 6**). Interestingly, this phenomenon was also discovered to be true in MiaPaCa (Pancreas) and PC3 (Prostate) cancer cells (**Supplemental Figures 12 and 13**).

Discussion

Normal cells are under continuous bombardment from intracellular and extracellular oxidative stress in the form of ROS (198). Damage caused by uncontrolled oxidative stress from lethal levels of ROS can lead to DNA strand breaks, mutation events and even cell death (199). Thus, mechanisms that facilitate control over ROS levels are uniquely important to cell proliferation and survival. Importantly, normal levels of ROS are needed in various cell-signaling events involving cell proliferation as well as programmed cell death. It is also of note that specific ROS are critical to many disease processes such as aging and cancer (198, 199). Thus, various defense mechanisms have evolved to regulate exposure to endogenous and exogenous ROS. These mechanisms include the transcription factor Nrf2 that transcriptionally activates the expression of numerous down stream target genes that modify and regulate the duration and exposure level to ROS (200). The genes activated by Nrf2 include glutathione peroxidase, catalase and *NQO1*. The down stream targets of Nrf2, such as *NQO1*, regulate exposure to ROS from both exogenous and endogenous sources and play a critical regulatory role in cell survival and cell death.

Cancer cells, just as in normal cells, must regulate ROS levels and have adapted to exposure to high levels of ROS through the altered expression of specific ROS regulatory genes that aid in their survival (201). Catalase for example, is normally expressed at high levels in normal tissues, however in tumors its levels are relatively low (202). The down-regulation of catalase expression in tumors is not clearly understood. However, catalase suppression has been associated with specific tumor promoting signaling pathways and resistance to chemotherapeutics (169, 203). Interestingly, studies with breast cancer and lung cancer cells have shown that re-expression of catalase modifies their exposure to ROS levels from their tumor microenvironment and ultimately enhances tumor cell death (204, 205). This would imply that reversing the expression of specific ROS regulatory genes in cancer cells could potentiate a tumor specific cell death.

Previous studies have implicated *NQO1* as a prognostic marker that negatively affects patient survival (137, 206). In most of these studies, the poor patient outcome is attributed to the existence of 2 prominent *NQO1* polymorphisms, referred to as *2 and *3. The *2 mutation is more common and involves a C to T point mutation at nucleotide position 609. These polymorphisms exist at varying, but small percentages, within the population (171). Those patients whose tumors were identified to have the homozygous *2 mutation in *NQO1*, were found to be more susceptible to issues involving chemotherapeutic toxicity when exposed to *NQO1*-detoxified therapeutics such as epirubicin (137). In contrast, recent retrospective analyses have shown that elevated *NQO1* expression in patient tumor versus normal tissue predicts poor patient survival (185, 186). We hypothesized, from the latter studies, that tumors elevate *NQO1* to enhance survival and that reduction of *NQO1* could potentially ameliorate the negative effects of tumor-*NQO1* overexpression on patient outcome.

In the current study we focused on determining whether decreasing elevated tumor-*NQO1* levels in lung cancer cells would inhibit tumor survival. We chose specific readouts, such as anchorage-independent growth, anoikis and *in vivo* tumorigenesis

assays, to make a connection between tumorigenic processes and the role that *NQO1* played in each. Our data clearly show that loss of *NQO1*, by stable shRNA knockdown, significantly affected anchorage- independent growth of lung cancer cells, which is hallmark of tumorigenesis. These data suggested that *NQO1* overexpression is intimately involved in the survival and proliferative capacity of lung cancer cells that overexpress *NQO1*. These data were corroborated by dicoumarol and Mac220 studies that showed that treatment with *NQO1* inhibitors significantly decreased the growth of lung cancer cells in soft agar. In addition to inhibiting growth in soft agar, we showed that loss of *NQO1* potentiated anoikis, suggesting that cells that were *NQO1* depleted were more susceptible to detachment induced cell death. This was further supported by the increase in ROS that was found in shNQO1 cells versus our control cells which correlates with increased anoikis. We also showed that loss of *NQO1* decreased both cell proliferation and invasion suggesting that knocking down *NQO1* decreases the tumorigenic potential of lung cancer cells. In contrast to cancer cells, our *in vitro* data show that transient knockdown of *NQO1* in non-transformed, non-tumorigenic human bronchial epithelial cells (HBECs) did not reduce their short-term viability or long-term survival (**Supplemental Figure 14**).

Our *in vivo* studies confirmed that stable *NQO1* depletion increased long-term survival in mice since shNQO1 tumors were significantly smaller than control tumors, and survival of mice bearing shNQO1 tumors was significantly enhanced as compared to mice bearing control tumors. Finally, we show that loss of *NQO1* substantially reduced the ALDH^(high) population in lung, pancreas and prostate cancers. In previous reports it has been demonstrated that ALDH^(high) activity within a tumor population is a reliable marker for cells that have a cancer stem cell phenotype in a number of malignancies (196, 197). Interestingly, knockdown of specific ALDH isoforms has been linked to the loss of stemness and tumorigenicity in lung cancer (207). Our studies show that loss of *NQO1* reduces the population of cells with ALDH^(high) activity, suggesting that the loss in tumorigenicity seen in *NQO1* knockdown cells (**Figure 5 C-E**) is attributable to the loss of the

ALDH^(high) subpopulation of cells (**Figure 6**). In summary, we report for that *NQO1*, a gene found overexpressed in many solid tumors, including NSCLC, can be directly targeted for therapy since it plays a critical role in the overall growth, invasive potential and survival of lung cancer. We hypothesize that *NQO1* expression is increased in tumors to thwart ROS stress, and that reversing the elevated expression of tumor-*NQO1* leads to increased susceptibility to ROS and reduced tumor burden due to anoikis and the loss of the ALDH^(high) cell population (**Figure 7**). These results suggest that *NQO1* depletion may be an important link in eliminating cancer stem cell populations not only in lung cancer, but in other malignancies as well. Finally, these data establish the potential for a new clinical approach that targets *NQO1* in lung cancer patients whose tumor-*NQO1* expression levels are often found to be 5-20 times more elevated than the *NQO1* levels in their adjacent normal lung tissue(170)

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Supplemental Figures

Supplemental Figure 2. shNQO1-B knockdown of *NQO1* leads to decreased growth in soft agar. In **A**, Western blot for *NQO1* expression in A549 cells stably knocked down for *NQO1* using lentiviral (shNQO1-B) and compared to A549 parental, lentiviral vector control (shCtr-L) and A549 sh*NQO1* (retroviral) knockdown cells. In **B**, A549-shCtr-L and A549-shNQO1-B cells were subjected to soft agar assays as described in "Materials and Methods". The soft agar data presented here are represented in graphical form in Figure 2 C of the manuscript.

Supplemental Figure 3
H292

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shCtr-L **shNQO1-B**

Supplemental Figure 3. shNQO1-B knockdown of *NQO1* in H292 cells leads to decreased growth in soft agar. H292-shCtr-L and H292-shNQO1-B cells were subjected to soft agar assays as described in “Materials and Methods” for H292 cells. The soft agar data presented here are represented in graphical form in Figure 2 D of the manuscript.

Supplemental Figure 4

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Supplemental Figure 4. Dicoumarol inhibits growth of A549 cells in soft agar. In **A**, Western Blot for *NQO1* expression in A549 cells exposed to 50μM dicoumarol for 2 or 24 hours. A549 sh-NQO1 cells were used as negative control. In **B**, following dicoumarol treatment, cells were liberated by trypsin, enumerated and subjected to soft agar assays as described in "Materials and Methods". In **C**, quantitative representation of soft agar plates that were imaged after 3 weeks using an Epson V700 photo scanner. The enumeration of colonies present in each dish was quantified using ImageJ software.

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Supplemental Figure 5. Mac220 inhibits growth of A549 cells in soft agar. In **A**, to demonstrate the effect of Mac220 on an NQO1-dependent chemotherapeutic, A549 cells were treated for 2 hours with varying doses of ARQ-761 (aka β -Lapachone, B-Lap) in the presence or absence of the NQO1 inhibitor dicoumarol (DIC, 50 μ M) or Mac220 (5 μ M). After 2 hours, drug media was removed and fresh media was added. Cells were allowed to grow for 7 days prior to evaluation of survival using a Hoescht DNA content assays as described previously (9,12). In **B**, following 24 h treatment with 50 μ M dicoumarol or 5 μ M Mac 220, A549 cells were liberated by trypsin, enumerated and subjected to soft agar assays as described in "Materials and Methods". In **C**, quantitative representation of soft agar plates that were imaged after 3 weeks using an Epson V700 photo scanner. The enumeration of colonies present in each dish was quantified using ImageJ software.

Supplemental Figure 6. Stable overexpression of *NQO1* in H596 (*NQO1* null) lung cancer cells causes increased growth in soft agar. In **A**, Western Blot analysis of *NQO1* null H596 cells that were stably transfected with *NQO1* using an LPC-*NQO1* retro viral construct or vector control (LPC-X) as described previously (9). In **B**, *NQO1* enzyme assays were performed according to the manufacturers protocol described in "Materials and Methods" Shown is a representative quantification of *NQO1* enzymatic activity performed in triplicate. In **C**, cells were liberated by trypsin, enumerated and subjected to soft agar assays as described in "Materials and Methods". In **D**, quantitative representation of soft agar plates that were imaged after 3 weeks using an Epson V700 photo scanner. The enumeration of colonies present in each dish was quantified using ImageJ software.

A**H596** **B** **C**

Supplemental Figure 7. Transient knockdown of *NQO1* in H596 LPC-NQO1 cells causes decreased growth in soft agar. In **A**, Western Blot analysis of H596 LPC-NQO1 cells that were transiently transfected with *NQO1* siRNA or scramble control siRNA and harvested after 48 h as described in “Materials and Methods”. In **B**, following transient transfections cells were liberated by trypsin, enumerated and subjected to soft agar assays as described in “Materials and Methods”. In **C**, quantitative representation of soft agar plates that were imaged after 3 weeks using an Epson V700 photo scanner. The enumeration of colonies present in each dish was quantified using ImageJ software.

Supplemental Figure 8 Madajewski et al

Supplemental Figure 8. shNQO1-B knockdown of *NQO1* in A549 cells decreases cell invasion. In **A**, A549 shCtr-R and A549 shNQO1 cells were allowed to form spheroids and the spheroids were placed atop of noble agar. The total area was measured after 10 days of culture. In **B**, A549-shCtr-L and A549 shNQO1-B cells were subjected to invasion assays as described in "Materials and Methods". Images and quantitation of invasion distance was performed as described in "Materials and Methods".

Supplemental Figure 9

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A

B

Supplemental Figure 9. Stable overexpression of *NQO1* in H596 *NQO1*-null cells causes increased invasion. In **A**, H596 LPC-X and H596 LPC-NQO1 cells were allowed to form spheroids and the spheroids were placed atop of noble agar. Spheroids were then subjected to invasion assays as described in "Materials and Methods". In **A** and **B**, Images and quantitation of invasion distance was performed as described in "Materials and Methods".

Supplementary Figure 10. Transient knockdown of *NQO1* using siRNA inhibits invasion of HCC1171 cells. In **A**, HCC1171 lung cancer cells were transiently transfected with control siRNA or *NQO1* siRNA as described in "Materials and Methods". After 48 h cells were harvested for Western Assays to detect *NQO1* expression. In **B**, following transfection cells were enumerated and subjected to invasion assays as described in "Materials and Methods".

A549

Supplemental Figure 11. Stable knockdown of *NQO1* in A549 cells causes loss of *PARP-1* protein expression. Western Blot analysis of A549 shCtrl-R and shNQO1 tumors harvested from mice at various times in long-term survival studies (Figure 5 D). Samples were harvested in PARP-lysis buffer as described in "Materials and Methods" and probed for *PARP-1* and *NQO1*. Blots were also probed with β-actin to determine loading equivalence.

Supplemental Figure 12
MiaPaCa

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Supplemental Figure 12. Knockdown of *NQO1* reduces ALDH^(high) activity in Pancreas cancer cells. In **A**, representative flow cytometry tracing of MiaPaCa shNQO1 knockdown cells and shCtr-R cells analyzed for ALDH^(high) activity using the Stem Cell Kit from "Stem Cell Technologies". Cells were assayed according to manufacturers protocol as described in "Materials and Methods". DEAB was used as an inhibitor of ALDH^(high) activity. In **B**, graphical representation of shNQO1 and shCtr-R cells assayed for ALDH^(high) activity. Graph represents experiments repeated at least 5 times in duplicate.

Supplemental Figure 13

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PC3

Supplemental Figure 13. Knockdown of *NQO1* reduces ALDH^(high) activity in Prostate cancer cells. In **A**, representative flow cytometry tracing of PC3 shNQO1 knockdown cells and shCtr-R cells analyzed for ALDH^(high) activity using the Stem Cell Kit from “Stem Cell Technologies”. Cells were assayed according to manufacturers protocol as described in “Materials and Methods”. DEAB was used as an inhibitor of ALDH^(high) activity. In **B**, graphical presentation of shNQO1 and shCtr-R cells assayed for ALDH^(high) activity. Graph represents an experiment performed in triplicate.

Supplemental Figure 14. NQO1 knockdown does not inhibit short-term viability or long-term survival of non-transformed human bronchial epithelial cells. In **A**, high levels of NQO1 activity are detected in A549 cells as compared to the low levels detected in the non-transformed, non-tumorigenic human bronchial epithelial cells (HBECs) using an NQO1 activity kit (ABCam) as described in "Materials and Methods". In **B**, Western-blot analyses of HBECs for NQO1 expression following a 48h transient transfection with scramble control siRNA or siRNA-NQO1 (Santa Cruz Biotech). In **C** and **D**, representative images of soft agar assays comparing tumorigenic A549 cells and non-transformed and non-tumorigenic HBECs (performed in triplicate) and quantitation of the colonies detected. In **E**, HBECs were subjected to transient transfections with siRNA-Control or siRNA-NQO1 (SantaCruz Biotech). After 48h, cells were enumerated and seeded at a density of 20,000 cells/well in 96 well plates for 24 h. The following day short-term viability assays were performed using CellTiter-Glo reagent (Progenia). Luminescence was detected using a Synergy-H1 Hybrid microplate reader. Relative Luminescence Units were calculated by subtracting the blank (media alone) wells from the test wells. In **F**, HBECs were subjected to transient transfections with siRNA-Control or siRNA-NQO1. After 48 h cells were seeded in 48 well dishes at a density of 2500 cells/well. Cells were allowed to grow for 7 days. After 7 days, long-term survival based on DNA content was detected using a Hoescht staining fluorescence assay described in "Materials and Methods". Fluorescence was detected using a Synergy-H1 Hybrid microplate reader. Relative Fluorescence Units were determined by subtracting blank wells containing Hoescht only from test wells.

Chapter 3

***NQO1* expression promotes the cancer stem cell phenotype in non-small cell lung cancer[#]**

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Running Title: *NQO1* expression promotes cancer stem cell-like characteristics

Key Words: *NQO1*, cancer-stem cells, stem cells, tumor initiating cells, chemoresistance, non-small cell lung cancer, proliferation, spheroids.

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Abstract

Cancer stem cells (CSCs) are purportedly a key resistance factor in most solid tumor models. Stem cell markers for heterogeneous bulk tumors have been loosely defined in the literature for most tumor types with albeit some consensus as to which assays are useful in determining whether specific genes are playing roles in the cancer stem cell phenotype. In the current study we utilized a tumor spheroid model to determine whether NADPH quinone oxidoreductase-1 (*NQO1*) was requisite in the promotion of the cancer stem cell phenotype in non-small cell lung cancer (NSCLC). Our data show that stable depletion of *NQO1* in A549 and H358 human NSCLC tumor models alters their ability to form primary tumor-spheroids. In addition to inhibiting primary spheroid formation, the loss of *NQO1* also affected serial, secondary and tertiary, spheroid formation. Interestingly when *NQO1* expression levels were rescued the spheroid formation ability of tumor cells was restored. Our data also show that A549 and H358 tumor spheroids were significantly less proliferative when *NQO1* was depleted as compared to control cells. Finally, our data also show that cisplatin refractory A549 tumor spheroids were rendered significantly sensitized to cisplatin due to *NQO1* depletion, suggesting that removal of *NQO1* reduces the stem cell resistant population. In summary, the data from these studies, along with our previous findings that show *NQO1* depletion reduces ALDH^{high} activity, strongly support a role for *NQO1* as marker for the CSC phenotype in NSCLC.

Introduction

Over the past two decades lung cancer has been the leading cause of cancer related deaths in the U.S. and worldwide (208). In 2016 it is estimated that more than 158,000 deaths will occur in the U.S. due to cancer of the lung and bronchus (209). Of the 221,000 estimated new lung cancer cases that will occur in 2016, 20% will be diagnosed as small cell lung cancer (SCLC), with the remaining 80% developing non-small cell lung cancer (NSCLC) (209-211). Both NSCLC and SCLC have abysmal 5-year survival rates of 15% and 5 % respectively (208, 209). The major factors leading to the poor survival rates observed in lung cancer patients include chemo-resistant disease, late stage diagnosis and subsequent spreading of disease (212, 213).

Interestingly, a number of NSCLC cases have demonstrated that overexpression of cytoprotective genes reduces the effects of commonly prescribed chemotherapeutics and radiation therapy (146). Of those genes with increased expression, NAD(P)H:quinone oxidoreductase 1 (*NQO1*), has been found to be overexpressed in ~60% of all NSCLC cases (117, 214). Additionally, the presence of lung cancer stem cells (CSCs) has become increasingly appreciated as a mechanism to enhance the tumorigenic properties of NSCLC. The ability of CSCs to initiate tumorigenesis, circumvent conventional therapies, and metastasize to distant locations makes targeting CSCs a promising strategy in the fight to improve patient survival.

NQO1 is a phase II detoxifying enzyme that is responsible for the neutralization of dangerous intracellular quinones, and in general serves to scavenge reactive oxygen species (ROS) (192, 215). Recently, there has been increased attention given to *NQO1* overexpression in a number of tumor malignancies as it has been shown to be a useful therapeutic target. Treatment with the quinone analog, β -lapachone (currently ARQ-761 in clinical trials), has shown promise inducing death in a tumor-specific manner. ARQ-761 enters into a futile cycle with *NQO1* that leads to ROS accumulation, poly ADP ribose polymerase hyperactivation, nucleotide depletion, and ultimately cell death (170, 175, 216). While substantial data exists to support *NQO1* overexpression as a valid drug target, the mechanism by which *NQO1* promotes the tumorigenic phenotype is not well understood.

In 1997, Bonnet and Dick were the first to describe the concept of a cancer stem cell when they demonstrated that a small leukemic cell population, defined by a set of cluster differentiation markers ($CD34^+CD38^-$), were able to establish the disease at low numbers in comparison the whole tumor population (217). Since the original identification of a leukemic CSC, researchers have discovered and defined CSCs in a number of other malignancies including breast, brain, and prostate cancers (218-220). To this point, a number of CSC markers have been used in an attempt to define the CSC population in NSCLC including *CD133*, aldehyde dehydrogenase (ALDH), and side population (221-223). Of the large number of markers used to define NSCLC CSCs, ALDH activity has shown the most promise in defining the small tumorigenic population, and because of this it has become the focus of a great amount of CSC research.

In an attempt to better understand *NQO1*'s role in tumorigenesis and tumor maintenance, our laboratory has begun to investigate the relationship between *NQO1* and maintenance of the cancer stem cell-like phenotype (**Figure 1**). Previously, we published that *NQO1* is important for a number of malignant characteristics including tumorigenesis, anoikis resistance, invasion, and even regulation of ALDH activity (224).

To expand upon the novel findings that showed the activity of the stem cell marker ALDH was reduced, suggesting less cancer stem-like cells, we have conducted new studies that link *NQO1* as having a vital role in the CSC phenotype of NSCLC. Here, we

demonstrate that *NQO1* is necessary for *in vitro* serial spheroid formation, therapeutic resistance, and *in vitro* limiting dilution tumor formation. Our data demonstrate for the first time that *NQO1* is vital to the maintenance of the CSC population and that reducing *NQO1* expression levels in tumors may prove to be a useful therapeutic approach for reducing chemo-resistant NSCLC CSC.

Materials and Methods

Cell Culture

A549, H358, and H596 cell lines were grown in Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine. Cell lines were cultured at 37° C with 20% oxygen and 5% carbon dioxide prior to spheroid culture. Cells were passaged weekly and supplemented with fresh media.

Stable *NQO1* protein knockdown

A549 and H358 cell lines underwent stable *NQO1* knockdown as previously described (224). Briefly, the sh*NQO1* retroviral vector was purchased from Open Biosystems (RHS1764-9691437), and lentiviral particles were purchased from Santa Cruz Biotechnology (sc-37139). The stable knockdown cell lines (sh*NQO1*, sh*NQO1* [lenti]) and empty vector controls (shCtr, shCtr [lenti]) were created for both A549 and H358 cell lines by viral infection in polybrene supplemented media containing either sh*NQO1* or shCtr viral particles. Following viral infection, cells were then put in limited dilution under puromycin (2 µg/ml) selection, and screened for *NQO1* expression via

Western blot. A549-shNQO1 and H596 cell lines were forced to express *NQO1* via the retroviral vector (LPC-NQO1) or the empty vector control (LPC-X) as previously described. Cells were then put into limited dilution and evaluated for *NQO1* expression via Western blot.

Western blotting

Protein lysates were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with 5% milk in PBST for 1 hour at room temperature, and then incubated overnight with β -actin (1:5000, Santa Cruz Biotechnology) at 4°C. Blots were washed in PBST and incubated for 1 hour with 1:5000 dilution of goat-antimouse IgG-HRP in 5% milk in PBST. The process was repeated using a 1:5000 dilution of monoclonal *NQO1* antibody (clone A-180, Santa Cruz Biotechnology), as well as 1:1000 dilutions of monoclonal *Shh*, *SOX2*, and *Nanog* antibodies (Cell Signaling). Pierce ECL western blotting substrate (Thermo Scientific) was used to visual bands on Hyblot-CL autoradiography film (Denville Scientific)

NQO1 Activity Assay

NQO1 enzyme activity was performed as previously described (224). Briefly, 2×10^7 cells of each cell line were collected. Pellets were solubilized in extraction buffer for 20 min, after which they were centrifuged at $18,000 \times g$ for 20 min at 4° C. Supernatants were collected into eppendorf tubes at stored at -80° C. Samples were then run according to the manufacturers protocol for the *NQO1* activity assay kit from Abcam. Results were read at an absorbance of 440 nm every 20 seconds for 5 minutes utilizing the Synergy-H1 Hybrid microplate reader. Plates were shaken both before and after each reading.

Spheroid Formation

Low-attachment culture plates were produced by coating the plates (Corning) with a 0.2% poly-hema/95% ethanol solution. Plates were incubated at 60° C overnight and allowed to dry. The process was then repeated a second time. Plates were washed twice with milli-Q water immediately prior to use. Cells were trypsinized and treated with trypsin neutralizing solution (1:1 ratio) prior to being counted using a hemocytometer. 160,000 cells were then plated in the low attachment 150 mm plates in 0.25% FBS DMEM supplemented with 1% L-glutamine. Cells were allowed to form spheroids over 14 days, at which time they were collected, trypsinized into single cell suspensions, and utilized for the respective assay. For spheroids grown in methylcellulose suspension, 1% methylcellulose in 0.25% FBS DMEM was further diluted in 1:1 0.25% FBS DMEM and cells were added to this mixture. The cell suspension was then plated on low attachment plates and imaged 2 weeks later for quantification.

Extreme limited dilution assay

Low attachment 96-well plates were prepared by treating plates with 0.2% poly-HEMA in 95% ethanol and allowing them to dry overnight. This process was repeated a second time to ensure proper application. Prior to plating the 96-well plate was washed twice with sterile milli-Q water. Cells were trypsinized, counted, and plated in 0.25% FBS-containing DMEM at densities of 40, 120, 360, and 720 cells per well. Each dilution

was performed in 24 wells. Cells were allowed to expand over three weeks (21 days), at which time the wells were examined for the presence of spheroids. A well containing a spheroid was counted as one, multiple spheroids per well did not increase the number of positive wells. The number of positive wells per dilution was then entered into the extreme limited dilution cancer stem cell frequency calculating software available at <http://bioinf.wehi.edu.au/software/elda/>.

Drug Treatment Studies

Spheroids were collected after 14 days and trypsinized into single cell suspension. Cells were counted and suspended in DMEM media supplemented with 0.25% FBS and 1% L-glutamine at a concentration of 10,000 cells/ml. 200 μ l of cell suspension was then added to each well. Cells were allowed to attach overnight, and the following day were treated with 0, 2.5, 5, and 10 μ M cisplatin dissolved in DMSO. Stock concentrations of 2.5, 5, and 10 mM cisplatin were diluted 1:1000 in 0.25% FBS DMEM supplemented with 1% L-glutamine and incubated on cells overnight. Each dose was performed in 8 replicates for each dose tested. Twenty-four hours after initial treatment, cell viability was assessed using Cell-Titer Glo (Promega), according to the manufacturer's protocol utilizing the Synergy-H1 Hybrid Reader.

Cell Proliferation Assays

Spheroids were mechanically (pipetting) and enzymatically (trypsin) broken down into single cell suspension. Cell suspension was then treated 1:1 with trypsin neutralizing solution to inactivate the trypsin. Cells were then quantified using a hemacytometer and suspended in 0.25% FBS DMEM at a concentration of 10,000 cells/ml. Utilizing 96-well plates, 100 μ L of each cell suspension was plated in 5 wells, and the respective plate was collected at the time points of 0, 24, 48, and 72 hrs. At this time 1e6 cells was also collected in a micro-centrifuge tube in order to later generate a standard curve. Collected plates were washed once with PBS, aspirated, and froze at -80° C, until all plates were collected and ready to be read. Plates were quantified using CyQuant Cell Proliferation Assay (Thermo Fisher), according to the manufacturers protocol utilizing the Synergy-H1 Hybrid Reader.

Quantitative real-time qRT-PCR

Total RNA was isolated utilizing the Trizol extraction method (225). cDNA was created from the total RNA sample utilizing iScript cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed using primers designed for *NQO1* (Fwd- 5'-CCAGATATTGTGGCTGAACAAA-3'; Rev- 5'-TCTCCTATGAACACTCGCTCAA-3'), and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Samples were analyzed using the CFX Connect Real-time qRT-PCR System (Bio-Rad). Relative expression values were calculated utilizing double delta C_t analysis.

Statistical analysis

Data analyses were performed using GraphPad Prism 6 software. Statistical significance was determined by using the Student *t* tests, and *P* values from these analyses were reported. Differences were considered significant when *P* values were < 0.05.

Results

NQO1 is essential for *in vitro* spheroid formation

In order to evaluate the stemness of a cancer cell population, it is common to utilize the spheroid formation assay as it demonstrates the ability of cells to replicate in a detached environment, one of the hallmarks of the transformed phenotype (46, 226-228). To determine the role of *NQO1* in stemness using spheroid assays as an

assessment tool, *NQO1* levels were reduced in both the A549 and H358 NSCLC cell lines via the stable retroviral expression of shRNA toward *NQO1*, (**Figures 2A & 2C**) respectively.

As expected from our previous experience in creating stable *NQO1* knockdown cell lines, the reduction in *NQO1* protein expression correlated with a decrease in *NQO1* activity (**Figures 2B & 2D**).

Subsequently, we demonstrated that the reduction of *NQO1* protein levels leads to a near total loss in primary spheroid formation in A549 (**Figure 3A**) and H358 (**Figure 3C**) cell lines. A549-shCtrl cells were able to form ~30 spheroids per field of view (50X magnification), where the A549-shNQO1

population demonstrated a significantly reduced ability to produce spheroids (**Figure 3B**). Interestingly, the inability of A549-shNQO1 cells to form spheroids was not a result of significant cell death, as trypan blue exclusion assays and analysis of apoptotic endpoints (PARP-1 cleavage and AIF expression), show

no significant difference over time (**Supplemental Figure 1**). Additionally, this data was recapitulated using a second, lentiviral driven shRNA toward *NQO1* with similar results (**Supplemental Figure 2A**). The H358 cell line does not appear to have a robust sphere forming ability as compared to A549 cells as they form fewer spheres per field of view. However, a significant difference exists between the H358-shCtrl and H358-shNQO1 populations (**Figure 3D**). To confirm that our spheroids were forming because of clonal expansion and not forming due to aggregation, we also formed spheroids using methylcellulose and found a similar significant difference in spheroid forming ability in A549 cells that were depleted in *NQO1* expression versus controls (**Supplemental Figure 2B**). In an effort to further confirm *NQO1*'s importance in spheroid formation, the

NQO1 inhibitor, dicumarol, was added to the media of A549-shCtrl and H358-shCtrl cultures and monitored for sphere formation (**Supplemental Figure 3**).

In addition, we validated the spheroid assay as a method by which to enhance the CSC population through the evaluation of the known stem cell markers, *Sox2*, *Shh*, and *Nanog*. We determined that spheroid culture increased the expression of each marker in comparison to normal (attached) culture conditions, that was subsequently decreased following differentiation culture conditions in 10% FBS containing media (**Supplemental Figure 4**). These results thus validate the spheroid culture method as a means to enhance the CSC population *in vitro*.

Interestingly, when we evaluated the expression of *NQO1* in spheroids, we noticed the expression of *NQO1* protein in the A549-shNQO1 population remained reduced in comparison to A549-shCtrl spheroid cells (**Supplemental Figure 5A**), however there was an increase in *NQO1* mRNA in the A549-shNQO1 population, as determined by real-time qRT-PCR (**Supplemental Figure 5B**). The level of *NQO1* mRNA expression remained significantly

reduced in comparison to the A549-shCtrl cell line, however a significant increase was noted in comparison to A549-shNQO1 cells plated in 2D culture (A549-shNQO1 2D). These results indicated that *NQO1* expression may be vital to spheroid formation, as those A549-shNQO1 cells that are capable of forming spheroids have a robust increase in *NQO1* expression in comparison to A549-shNQO1 cells grown in attached conditions. Additionally, this data demonstrate that the spheroid culture enhances the CSC population, and that *NQO1* reduction severely inhibits the ability of NSCLC cell lines to initiate spheroid formation.

***NQO1* is necessary for serial *in vitro* sphere formation**

CSC properties include the ability to divide asymmetrically in order to continually produce both a CSC population as well as a population of proliferative progenitor cells (229). This allows for the CSC population to continually perpetuate its tumor-initiating capabilities following gross reduction of total tumor cell numbers. In an effort to demonstrate the presence of CSCs within the A549 and H358 cell lines, serial tumor sphere formation assays were performed. Primary spheres were collected and single cell suspensions were made that were then placed back into the sphere forming assays. This process was carried out until tertiary spheres were formed. Given that both the A549-shNQO1 and H358-shNQO1 cells were severely hindered in their primary sphere

formation, it was not surprising that their ability to form secondary and tertiary spheres was also restricted (**Figures 4A & 4B**, respectively). In the case of the A549 cell line, secondary and tertiary A549-shNQO1 spheres formed at significantly lower numbers than A549-shCtr cells. These data clearly show that there exists an essential role for *NQO1* in serial perpetuation of tumor spheres. This finding is further supported by data obtained from the H358 cell line. H358-shCtr cells show a slight increase in the sphere forming ability upon serial plating, where H358-shNQO1 lose tertiary sphere forming abilities, a more supportive finding for *NQO1*'s role in serial sphere formation than what is seen in A549 cells. Interestingly, in a second H358-shNQO1 clone that has greater expression of *NQO1*, primary sphere formation remains equal to what is seen in controls (**Supplementary Figure 6**). These data suggest the possibility that a threshold of *NQO1* expression exists which allows serial formation of tumor spheres.

In addition to serial spheroid assays, *NQO1* rescue experiments were performed to definitively demonstrate *NQO1*'s necessity in spheroid formation. A549-shNQO1 cells were forced to express *NQO1* via retroviral vector, or the empty vector control. It can be seen in **Figure 4C**

that the level of *NQO1* expression is comparable to parental A549 cell line (left panel), and the re-expression of *NQO1* results in a significant increase in spheroid formation in comparison to the control vector (**Figure 4D**). Furthermore, we utilized the *NQO1*-null NSCLC cell line, H596 that harbors the *2 polymorphism. We then drove the expression of *NQO1* utilizing the retroviral *NQO1* vector and evaluated the sphere forming ability in comparison to the empty vector control (**Figure 5**). These results recapitulate what was observed when *NQO1* levels were rescued in the A549 cell line that is the presence of *NQO1* significantly increases the sphere forming ability of H596 LPC-NQO1 cells in comparison to its H596 LPC-X (*NQO1*-null) control.

Limited dilution assays are often performed in order to determine the tumor initiating cell frequency within a cell population (228). In order to determine the frequency of CSCs within the A549 and H358 cell lines, both cell lines were placed in an extreme

Figure 4. Loss of *NQO1* expression reduces serial spheroid formation, and re-expression of *NQO1* rescues tumor-spheroid forming ability. The self-renewal capabilities of lung cancer stem cells were assayed via a serial spheroid formation assay. In **A**, A549-shCtr cells form similar numbers of spheroids in primary (black bars), secondary (light grey bars), and tertiary generations (dark grey bars), where the A549-shNQO1 cells form significantly fewer spheroids in each generation (* = $p = 0.0018$, # = $p = 0.0102$, $\infty = p < 0.0001$). The H358 cell line, as seen in **B**, shows similar results (* = $p = 0.0035$, # = $p = 0.0449$, $\infty = p < 0.0001$). In **C**, Western blot analysis of A549-shNQO1 cells transfected with the retroviral *NQO1* vector in an effort to induce re-expression of *NQO1* protein. In **D**, primary spheroid formation in *NQO1* re-expressing A549-shNQO1 cells. The rescued *NQO1* expression induced a significant increase in the formation of primary spheroids ($p = 0.0121$).

limiting dilution assay. Results from this assay demonstrate that in the A549 cell line, those cells in which *NQO1* is present have an approximate 12-fold increase in the presence of cancer stem cells in comparison to the shNQO1 cell line. This was also true for the H358 cell line however to a lesser degree (~ 2-fold) (**Table 1**). These results suggest that *NQO1* is vital to maintenance of the cancer stem cell population, which is in agreement with previously published data (224).

***NQO1* knockdown inhibits proliferation, increases chemotherapeutic resistance of NSCLC tumor spheroids**

Increased cellular proliferation is often observed in tumor cells and previous studies have indicated the rapid proliferative capacity in various lung cancer cells. However, we assessed the proliferative capacity cells that were no longer attached to plastic to determine if *NQO1* expression mattered in the cells ability to grow in 3D-tumor spheroid culture. Our results show that indeed *NQO1* expression mattered since tumor

spheroid shCtr cells were significantly more proliferative as compared to shNQO1 cells for both A549 and H358 cell lines (**Figures 6A and 6B**).

A hallmark of the CSC phenotype is the inherent resistance to chemotherapy. Given that *NQO1* has been described to protect against chemotherapy in malignant cells, as well as protecting in chemotherapy-induced toxicities in normal cells, we hypothesize that spheroids expressing *NQO1* will be protected against cisplatin induced cell death (146, 167, 230-232). In order to test this, spheroids were

	40 Cells	120 Cells	360 Cells	720 Cells	CSC Frequency
A549-shCtr	2	0	19	18	1/455
A549-shNQO1	0	0	2	3	1/5659
H358-shCtr	0	0	1	1	1/14609
H358-shNQO1	0	0	1	0	1/29580

Table 1. Cancer stem cell frequency in A549 and H358 cells with or without shNQO1 expression. Quantification of the number of cancer stem cells present in the cell populations that have been examined was carried out utilizing an *in vitro* extreme limited dilution assay. This assay requires plating limited dilutions of cells in low attachment conditions, and examining wells for spheroid formation. Wells with at least one spheroid were counted as a positive well for the corresponding dilution. The results were then analyzed using Extreme Limited Dilution Analysis (ELDA) software. The resulting CSC frequencies demonstrate a marked increase in the CSC population found in A549-shCtr and H358-shCtr in comparison to their respective shNQO1 cell lines.

dissociated into single cell suspension and plated at a density of 1,000 cells per well in a 96-well plate. The following day, the media was removed and media containing 0, 2.5, 5.0, or 10 μ M cisplatin was added to the cells. The treatment of tumor spheroids with cisplatin lasted 24 h, after which the Cell-Titer Glo kit was used to measure cell viability. The data presented in **Figures 6C and 6D** demonstrate that for both A549 and H358 spheres, the expression of *NQO1* protects against the cytotoxic effects of cisplatin at all doses administered. The control groups for both cell lines were used to normalize the data, as there was an observed increase in viability (greater than 100% viability, data not shown), which is most likely due to an increase in cell numbers. These data suggest that specific refractory subpopulations within bulk NSCLC tumors are resistant to chemotherapeutics in part due to *NQO1* expression levels, and that cisplatin-resistant populations of CSC within A549 and H358 bulk tumors could be reduced by *NQO1* depletion strategies.

Discussion

It is generally accepted that a tumor is a population of many diverse cells that have various roles in the survival of the heterogeneous bulk tumor (233, 234). One purportedly critical cell type/population within the bulk tumor is thought to be the tumor-initiating cell or CSC that gives rise to chemoresistant cell populations, and are thought to be responsible for latent disease resulting in tumor recurrence, metastasis and poor prognoses (235-237). This specialized population of cells is understood to have features similar to those of normal stem cells including the ability to repopulate an entire population of diverse cell types. Thus, CSCs allow the continual self-renewal and

propagation of the CSC population. Many scholarly endeavors have been made over the past thirty years to identify and characterize the CSC phenotype within each tumor type. These efforts have led to the development of new therapeutic strategies for treatment of various cancers (238-240).

The identification and dissociation of the cancer stem cell population from the bulk tumor population has been primarily accomplished using markers for stemness. Although no iron clad pattern has developed over the years to allow a cook book determination of which population of cells within a bulk tumor represent the CSCs for that tumor, many studies have developed protocols using various stemness markers as strategies to collect these unique cell population. In breast cancer the most common stemness markers used in the isolation of CSCs include CD44^{high} CD24^{low} populations as well as populations that express ALDH^{high} activity and SOX2 expression (241, 242). While in lung cancers ALDH^{high} activity, along with Notch expression have been more closely linked to CSC population in lung cancer patients whose tumors recur, metastasize and are refractory to therapy (196, 243).

In addition to the heavy utilization of CSC markers, various tissue culture and propagation methodologies have been developed to isolate or enrich for CSC populations. For example, the 3D tumor spheroid model has become a staple amongst assays used to study drug efficacy, as well as the CSC phenotype. The spheroid model is believed to be more representative of tumor growth *in vivo* and thus its utilization has expanded to involve drug efficacy studies as well as isolation of CSC for analysis (35, 244).

In our recent studies we showed that *NQO1* depletion in the general population of A549 and H292 NSCLC tumor cells correlated with loss of ALDH^{high} activity (224). We also found that *NQO1* depletion inhibited proliferation, invasion and growth *in vivo*. Those data were the first to indicate that tumor-*NQO1* levels were linked to tumorigenesis, and that *NQO1* may be associated with the CSC phenotype. In the current study we compared tumor spheroid forming ability in cells with or without *NQO1*. In both our sh*NQO1* knockdown models we found that *NQO1* depletion reduced the ability of NSCLC cells to form tumor spheroids and their subsequent ability to form secondary and tertiary spheroids was significantly inhibited by the loss of *NQO1*. Interestingly when *NQO1* was rescued in our *NQO1* knockdown cell lines tumor spheroid formation was restored. In addition to *NQO1* rescuing *NQO1* knockdown cell lines, we also show that establishing *NQO1* expression in *NQO1* null cell lines allowed for a significant increase in the spheroid forming ability of *NQO1* null NSCLC cell line H596.

In support of *NQO1* enhancing the CSC population, we demonstrate that spheroid culture enhances the expression of known stem cell markers SOX2, Shh, and Nanog. Interestingly, these results are observed in both our A549-shCtr and A549-sh*NQO1* cell lines. When evaluating the expression of *NQO1* in our spheroids we notice an increased, yet significantly less, expression of *NQO1* in our A549-sh*NQO1* cell line in comparison to control. This argues the point that in order for A549-sh*NQO1* to survive and expand under spheroid conditions, increased expression of *NQO1* is vital. We

postulate that the increased expression of *NQO1* in our knockdown line is the reason we observe spheroid formation in the A549-shNQO1 cell line and an increase in stem cell markers during spheroid culture. These data and the loss of ALDH^{high} activity in our previous work, strongly suggest that *NQO1* is a CSC marker for NSCLC.

Finally, we investigated the effect that *NQO1* depletion had on chemotherapeutic resistance using tumor spheroids. Our data show that shCtr spheroids were resistant to cisplatin while shNQO1 spheroids were sensitized to cisplatin treatment. These data suggest that depleting *NQO1* expression reduces the drug resistant cell population from the population of cells rendering them more sensitive to chemotherapy.

In summary our data provide a sound rationale for developing therapeutics for tumors that overexpress *NQO1* focusing on decreasing *NQO1* expression to eliminate the CSC population. Possible strategies may include tumor targeted siRNA strategies to decrease the *NQO1* overexpressing population *in vivo*.

Supplemental Figures

Supplemental Figure 1

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Supplemental Figure 1. Spheroid culture does not affect cell viability. In **A**, the viability of cells in spheroid culture was evaluated via trypan blue exclusion over the two-week culture period. There was no obvious difference in cell viability between the A549-shCtr and A549-shNQO1 cell lines at anytime over the course of observation. In **B**, cells in spheroid culture were evaluated for apoptosis induction by evaluating *PARP1* cleavage, as well as AIF induction. A549 treated with 0.5 uM staurosporin [A549 (0.5 μ M Stauro)] served as a positive *PARP1* cleavage positive control. There was no apparent induction of *PARP1* cleavage, as well as no obvious increase in AIF expression over the spheroid culture period.

Supplemental Figure 2

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Supplemental Figure 2. Loss of *NQO1* via lentiviral shRNA inhibits spheroid formation, and spheroid formation is a result of clonal expansion. In **A**, Western blot analysis of lentiviral-shRNA knockdown of *NQO1* [A549-shNQO1 (lenti)] in comparison to the A549 control cell line [A549-shNQO1 (lenti)] (left panel). In **B**, spheroid assay of A549-shCtr (lenti) and A549-shNQO1 (lenti) demonstrating a significant loss of spheroid formation with *NQO1* reduction (** = $p = 0.0066$). In **C**, A549-sh-Ctr and A549-shNQO1 cells were tested for clonal spheroid forming ability in the presence of 1% methylcellulose, diluted 1:1 in 0.25% FBS/DMEM, as an alternative to 0.25% FBS/DMEM medium. Data show significant loss in spheroid forming ability in the A549-shNQO1 cell line in comparison to control, similar to what was observed in experiments with low serum and DMEM only (** = $p = 0.0084$).

Supplemental Figure 3

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Supplemental Figure 3. Inhibition of *NQO1* activity results in loss of spheroid generating capabilities. In **A**, parental A549 cells were tested for their spheroid forming capabilities in both the presence and absence of the *NQO1* inhibitor, dicoumarol. Cells were plated for the spheroid assay and treated with either vehicle control, or 50 μ M dicoumarol and allowed to incubate for 2 weeks. Following incubation, the number of spheroids formed was enumerated. The inhibition of *NQO1* leads to a significant decrease in the sphere forming abilities of the A549 cell line. In **B**, the same experiment was carried out using the H358 cell line, in which similar results were obtained.

Supplemental Figure 4

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Supplemental Figure 4. Spheroid cultured cells increase expression of stem cell markers, and are lost upon differentiation. A549-shCtr and A549-shNQO1 cells were pelleted prior to spheroid plating (shCtr and shNQO1), following two weeks of spheroid culture (shCtr 1° Sphere and shNQO1 1° Sphere), and following one week of differentiation in 10% FBS containing DMEM in an attached setting (shCtr 1° Sphere differentiated and shNQO1 1° Sphere differentiated). Western blot analysis was performed in order to visualize the expression of known stem cell markers SOX2, *Shh*, and *Nanog*. It was observed that there was an increase in the stem cell markers as a result of spheroid culture in both the A549-shCtr and A549-shNQO1 cell lines. The stem cell marker expression was subsequently decreased following plating in differentiating conditions.

Supplemental Figure 5

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A

B

Supplemental Figure 5. Spheroid culture conditions induce *NQO1* expression in A549-shNQO1 cells. Both A549-shCtr and A549-shNQO1 cells grown in spheroid culture for 2 weeks were assayed for *NQO1* expression. In **A**, Western blot analysis demonstrating decreased expression of *NQO1* in A549-shNQO1 spheres in comparison to A549-shCtr spheres. In **B**, real-time qRT-PCR analysis demonstrating a marked increase in *NQO1* mRNA expression in spheroid cultured cells (A549-shNQO1) in comparison to A549-shNQO1 cells grown in attached conditions (A549-shNQO1 2D). (** = $p = 0.0009$) Additionally, it should be noted that the expression of *NQO1* mRNA in the A549-shNQO1 sphere cultured cells remains significantly decreased in comparison to the A549-shCtr sphere cultured cells. (** = $p = 0.0018$).

Supplemental Figure 6

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A

B

Supplemental Figure 6. Spheroid formation is dependent on *NQO1* expression. In **A**, *NQO1* expression was evaluated in the parental, shCtr, shNQO1, and 4C20 (second clone) H358 cell lines. The 4C20 clone expresses a greater amount of *NQO1* than that of H358-shNQO1, and therefore was evaluated for its sphere forming abilities in **B**. In **B**, is the quantification of the spheroid formation assay, including our 4C20 cell line. It can be seen that with an increase in *NQO1* expression there is an increase in spheroid formation, suggesting that a critical level of *NQO1* expression is necessary to support spheroid formation.

Chapter 4

Summary and Conclusions

Summary

The primary focus, for studies regarding cancer and *NQO1*, has been placed on exploiting *NQO1* as a tumor-specific drug target. The work to date in this research area has provided a strong base of preclinical data, from which several clinical trials are now being conducted. Despite the exhaustive interrogation into *NQO1* as a tumor-specific drug target, there has been little to no investigation into deciphering the antioxidant's role in the promotion and progression of cancer. The work presented in this dissertation probed into the role *NQO1* plays in (1) promoting tumorigenesis and (2) maintaining the CSC population in NSCLC. Our results help to fill in the knowledge gap on the supportive role *NQO1* overexpression is playing in tumor initiation and progression.

In Chapter 2, we present our initial study investigating whether *NQO1* plays a supportive role in tumor initiation and progression in NSCLC. We began with determining whether *NQO1* is necessary to maintain a transformed phenotype. This characteristic was assessed using a soft agar colony formation assay. This assay demonstrates the ability of transformed cells to grow in a detached environment, and it was observed that decreased expression of *NQO1* severely impacted colony formation (**Chapter 2, Fig. 2A-D**). These results indicate that, without increased expression of *NQO1*, a phenotypic change occurs in which the cells have a reduced ability to proliferate in anchorage-independent scenarios.

Next, the invasive capabilities of NSCLC cell lines were analyzed by performing the spheroid invasion assay. When spheroids were implanted within a collagen I matrix, it was observed that *NQO1* was once again playing a positive role in promoting tumor cell invasion. This characteristic was lost upon reduction of *NQO1* (**Chapter 2, Fig. 3A-D**). These results suggest that *NQO1* has a supportive role in the promotion of tumor cell invasion, and it can be postulated, although not shown in this study, that *NQO1* may be aiding in the formation of metastasis as invasion is a crucial step in the establishment of metastatic growths (245).

NQO1 has been reported to function as a reactive oxygen species scavenger, and it is well known that detachment from matrix can lead to increased reactive oxygen species production(31, 230, 246). Given that we observed near complete loss of colony formation in soft agar, we proceeded to evaluate the level of reactive oxygen species in our cell lines. We determined that under detached conditions cells with their full allotment of *NQO1* had significantly lower levels of reactive oxygen species in comparison to those in which *NQO1* expression was reduced (**Chapter 2, Fig. 4A,B**). In conjunction with these findings, we demonstrated that the detachment of NSCLC cells from matrix also resulted in an increase in anoikis, or detachment-induced cell death (**Chapter 2, Fig. 4C,D**). These results demonstrate the importance of *NQO1* in the

survival of NSCLC in detached environments, such as those encountered when disseminated cancer cells become circulating tumor cells.

Next, *in vivo* tumorigenicity was evaluated by limited dilution assays in immunocompromised mice. We observed that loss of *NQO1* reduced tumor burden and increased the overall survival of mice (**Chapter 2, Fig. 5C-E**). In an effort to better determine how *NQO1* is affecting the tumorigenic potential of NSCLC cells, we evaluated the activity of known CSC marker ALDH. Surprisingly, we found significant loss of ALDH activity following depletion of *NQO1* (**Chapter 2, Fig. 6**). These results demonstrate that *NQO1* may be promoting tumor formation by enhancing the CSC population. This is the first report, to our knowledge, that ties the expression of *NQO1* to the activity of a known CSC marker in any cancer type.

The data presented in Chapter 2 is the first report in which *NQO1* is evaluated for its pro-tumorigenic role in the establishment of NSCLC. For the first time, *NQO1* is described as having an integral role in the formation, and progression of cancer. While much has been done to target *NQO1* due to its overexpression, our data provide the first evidence that there may be alternative approaches to targeting tumor *NQO1* levels, mainly through the use of RNA interference (RNAi) technologies. RNAi technology has been utilized previously, however only now has its clinical implications come to light. Clinical application of this approach does not come easy however, as there are many issues that exist with stability, delivery, and safety (247). Despite these current roadblocks, continued research in this field furthers the application daily. This technique could be a worthwhile option to explore, given the results we present herein. Reduction of tumor *NQO1* levels may work to inhibit further tumor growth, as well as the ability of cells to survive during periods of detachment. Regardless of the known limitations of this approach, the possibility of designing novel useful therapeutics will continue to be of significant benefit to patients battling this grave disease.

Our results depicted in Chapter 2 drove us to further investigate the role that *NQO1* plays in maintaining the CSC population within NSCLC. Observing the loss of ALDH activity following depletion of *NQO1* expression indicated that *NQO1* might be altering the CSC population in NSCLC. CSCs have been reported to be responsible for tumor recurrence, resistance, and metastasis, and therefore further investigation into the role *NQO1* was playing to support this population was warranted (248). In order to investigate this possibility, we utilized a number of well-established CSC assays, and demonstrated that loss of *NQO1* leads to a decrease in the prominence of the CSC population.

Inquiry into *NQO1* functioning to support the CSC phenotype began with evaluation of *in vitro* tumor sphere formation. The spheroid assay evaluates the tumor initiating capabilities of a cell population. Transformed cells are defined by the ability to survive and proliferate in an anchorage-independent manner, as demonstrated by this assay (249). In addition, the media utilized contains ultra-low levels of fetal bovine serum (0.25%) that enhances the stem cell population by reducing the differentiation of stem cells to their progenitors (57). Upon evaluation of the A549 and H358 NSCLC cell lines,

we determined that reduction of *NQO1* led to a significant decrease in the ability of these populations to form spheroids. This result indicated that *NQO1* was supporting the growth and expansion of the NSCLC stem cell population (**Chapter 3, Figure 2**).

Spheroid formation demonstrated an enrichment in the CSC population in those lines with their complete complement of *NQO1*, however it is of paramount importance to demonstrate self-renewal when attempting to demonstrate the presence of a CSC population (250). To do so, primary spheroids were dissociated, both mechanically and enzymatically, and plated into secondary spheroid assays, followed by tertiary plating. In all scenarios, it was demonstrated that loss of *NQO1* led to decreased spheroid numbers, indicating that *NQO1* is playing a supportive role in maintaining the CSC pool within NSCLC (**Chapter 3, Figure 4**). Furthermore, we established *NQO1* re-expression in our A549-sh*NQO1* cell line, as well as the *NQO1*-null cell line H596, and demonstrated a significant increase in the number of spheroids formed in comparison to controls (**Chapter 3, Figure 4C,D & Figure 5**).

In order to quantify the number of tumor initiating CSC within our populations, we employed the *in vitro* extreme limited dilution assay (ELDA). The ELDA assay functions similarly to the spheroid assay, but utilizes limiting dilutions of cells, and software (available at <http://bioinf.wehi.edu.au/software/elda/>), to calculate the number of CSCs. The results from this assay demonstrated a greater than 10-fold enrichment in the A549 cell lines, as well as a doubling of the CSC population between the H358-shCtr and H358-sh*NQO1* cell lines. In addition, there was a greater than 30-fold enrichment between the A549-shCtr and H358-shCtr cell lines (**Chapter 3, Table 1**). This correlates well with an increase in the sphere forming abilities of the A549 cell line versus that which is seen in the H358 cell line (**Chapter 3, Figure 2**). The results of the ELDA assay, spheroid formation, and *NQO1* rescue experiments strongly back a supportive role for *NQO1* in the CSC population of NSCLC.

Finally, we evaluated the chemotherapeutic resistance of *NQO1* knockdown cells versus controls cultured as spheroids. CSCs are believed to be responsible for therapeutic resistance (18, 44, 87), and it is of great importance to demonstrate this trait when describing a CSC population. NSCLC spheroids were dissociated after two weeks in culture and plated as single cells. This assay was carried out in this manner to evenly treat all cells found within the spheroid. The spheroid model has been utilized as a drug treatment model of micrometastasis, and functions to determine the diffusion limit of drug across an avascular tumor (251). In addition, the CSC population has been described to be centrally located within these spheroids and thus protected from drug treatment (252). Single cell suspension was the only method by which to treat all cells of the spheroid in an unbiased manner. A549 and H358-shCtr cells, treated for 24 hours with increasing doses of cisplatin, displayed an increased survival over their *NQO1* knockdown counterparts (**Chapter 3, Fig. 6**). These results demonstrated that *NQO1* protected NSCLC cells from the deleterious effects of common, clinically utilized, anti-cancer therapeutics.

Conclusions

Lung cancer continues to be the leading cause of cancer related deaths in the world (2). This trend continues in spite of attempts to decrease lung cancer risk with approaches such as smoking cessation. Declines in the percentage of the smoking population in recent history have yet to result in significant decreases in lung cancer incidence (**Figure 1**) (253). These observations indicate that there are other contributors to the formation of lung cancer besides smoking, an example being pollution (254). Regardless of the extrinsic factors that are influencing lung cancer development, it is paramount to research and develop new approaches to treating those patients with the disease.

The modern age of anti-cancer therapy has gone personal. The idea of personalized medicine is one in which many believe is the future of cancer therapy (255, 256). Unveiling and understanding both new and old cancer-promoting pathways will reveal targets toward which to design new medications in an effort to thwart pro-tumorigenic signaling. A major shortcoming with this approach however, is the high rate of refractory disease (257). The eventual ineffectiveness of targeted drugs requires that secondary and tertiary lines of therapy be developed in an effort to prolong the positive response.

The CSC population is a unique population of cells that exist within each tumor purportedly having unique abilities including therapeutic resistance, the ability to seed at distant metastatic sites and to cause relapse of disease (32). The CSC populations have become highly researched due to the belief that obliteration of this population will lead to total eradication of tumors (258). In principal this idea is simple, but in practice it is quite the challenge. Currently, there are few therapies that are targeted toward CSCs, and often those therapies are ineffective or toxic to the patient (259, 260). An obvious major roadblock to CSC targeted therapy is the utilization of markers that are also present on somatic stem cells throughout the body (261). Eliminating the CSC population has the very real potential to have an off-target effect on the somatic stem cell population (262). From this perspective, it becomes clear that CSC targeted therapies must target pathways, or markers, that are specific to cancer.

NQO1 has repeatedly been demonstrated to be overexpressed in tumor in comparison to normal tissues (116, 122). This has lead to the development of *NQO1* targeted therapy that has progressed to clinic (263). This dissertation presents data that fills in the void on *NQO1*'s role in NSCLC. We demonstrate that the up-regulation of *NQO1* observed in tumors promotes the tumorigenic characteristics and progression of disease (**Chapter 2**). Additionally, we show a novel role for *NQO1* in the maintenance of the NSCLC CSC population (**Chapter 3**). These findings are important and impactful for a number of reasons including 1) furthering the understanding of *NQO1*'s mechanism of action in the promotion of NSCLC and 2) defining a novel stem cell maintenance pathway. The results presented here provide the groundwork for future studies that delve deeper into the workings of *NQO1* as a tumor-promoting factor, and argue that pharmaceutical approaches to reducing the tumor expression of *NQO1* may be a viable therapeutic strategy. Given that we demonstrate *NQO1* to have a positive role in supporting the CSC population, as well as tumor progression, the utilization of therapies to reduce *NQO1* expression, such as RNAi technology, could provide clinicians with a two-headed approach; one that can stop cancer at the source, and another that will reduce the malignant characteristics of the progenitor population.

Chapter 5

Preliminary Data and Future Directions

Preliminary Data

Our investigation into the role of *NQO1* in tumorigenesis and maintenance of the CSC population has utilized gene manipulation via short hairpin RNA (shRNA) technology. shRNA has provided investigators the ability to alter gene expression through the specific targeting of messenger RNA encoding their gene of interest (264). The formation of double stranded RNA through the binding of shRNA to the target mRNA, leads to the destruction of both the message and shRNA, thus reducing the expression of protein (265) (**Figure 1A**). The use of this tool has unveiled numerous discoveries, however the technology does not allow for the complete elimination of protein expression. In an effort to better understand the role that *NQO1* plays, utilization of the CRISPR-Cas9 system was incorporated into our ongoing studies. CRISPR-Cas9 allows for direct alteration of the genome that results in the complete loss of protein expression (266) (**Figure 1A**). For these reasons we employed the CRISPR-Cas9 system in our A549 NSCLC cell line in order to determine the phenotypic effects of total *NQO1* protein loss.

Studies were initiated with transfection of the lentiviral CRISPR-Cas9 system containing the small guide RNA (sgRNA) toward *NQO1* into the A549 cell line (**Figure 1B**). Following selection with puromycin (2 µg/ml), cells were plated in limited dilution in order to acquire single cell clones that were subsequently expanded. Once expanded, Western blot analysis was utilized to determine which, if any, clones had lost *NQO1* expression. We discovered two successful *NQO1* knockout clones (A549 C-NQO1 1 and A549 C-NQO1 2) as determined by Western blot, and

subsequent sequencing (**Figure 1C**, **Supplemental Figures 1-2**). We then performed an *NQO1* activity test in order to confirm loss of protein activity (**Figure 1D**). It was determined that there was no significant difference in *NQO1* activity between the parental A549 cell line (A549) and the CRISPR-Cas9 control (A549 C-Ctr), however there was complete loss of *NQO1* activity in the A549 C-NQO1 1 and A549 C-NQO1 2 cell lines. Absence of activity was confirmed by comparison to the *NQO1**2 cell line H596, which is known to lack functional *NQO1*. These results demonstrate that utilization of the CRISPR-Cas9 system resulted in the creation of A549 *NQO1*-null cell lines, the first of their kind to our knowledge.

We next investigated the effect of complete *NQO1* loss in a number of assays that we have previously employed. These included spheroid formation, soft agar colony formation, and drug resistance. Surprisingly there was no significant difference in the number of spheroids (**Figure 2A & B**) or colonies formed (**Figure 2C & D**). Additionally, no alterations in resistance to the platinum based chemotherapy cisplatin were noted between our A549 C-Ctr and C-NQO1 knockout cell lines (**Figure 2E**). These results were surprising given that our results with shRNA-NQO1 knockdown in A549 cells led to a significant decrease in all of these assays. In order to account for this difference, we compared the differences between the two methods. We concluded one method (shRNA) directly targets mRNA, whereas the CRISPR-Cas9 approach directly edits the genome and may result in the production of a non-translatable mRNA. This understanding led us to hypothesize that it is not *NQO1* protein, but the *NQO1* mRNA that is responsible for maintaining the observed phenotype.

We first evaluated the expression of *NQO1* mRNA in our A549 C-NQO1 cell lines (C-NQO1 1 and C-NQO1 2) versus the A549 C-Ctr. Results demonstrated that there was no alteration in the expression of *NQO1* mRNA in the A549 C-NQO1 in comparison to A549 C-Ctr. (**Figure 3A**) In an effort to reduce the expression of *NQO1* mRNA in the A549 C-

NQO1 1 cell line, we transiently knocked down the mRNA levels using siRNA directed toward *NQO1*. Significant reduction in the expression of *NQO1* mRNA was achieved in both the A549 C-Ctr and C-NQO1 1 cell lines, as determined by real-time qRT-PCR (**Figure 3B**). We next evaluated the sphere forming ability of the cells treated with siRNA toward *NQO1* (A549 C-NQO1 1 siNQO1) versus the scrambled control (A549 C-NQO1 siCtrl). Results demonstrate a significant decrease in the sphere forming ability, of those cells treated with siRNA toward *NQO1* (**Figure 3C**). Additionally, when measuring the size of spheres formed, a significant decrease in sphere area was observed in the siRNA treated cells versus those treated with control.

These results are the initial demonstrations of a novel role for *NQO1* mRNA, the regulation of the CSC phenotype in NSCLC. While these results are preliminary, they provide enough support to postulate a mechanism by which the *NQO1* mRNA is playing a signaling role in NSCLC. We hypothesize that *NQO1* mRNA is acting as a micro-RNA (miR) sponge for a possible number of known CSC associated miRs, including the Let-7 family, miR-34a, and miR-143 (267-269). (**Figure 4A**) Reduction of the listed miRs has been demonstrated previously to result in an increase in the CSC phenotype that would coincide with our observation that reduction of *NQO1* mRNA results in decreased prominence of the CSC phenotype, possibly thorough the liberation of the listed miRs (268, 270, 271). (**Figure 4B**) In addition, the miRs listed have been predicted to bind to *NQO1* mRNA according to the prediction software available at www.targetscan.org.

Future studies will be necessary in order to confirm the relationship of *NQO1* mRNA with that of the listed, and possibly greater numbers of miRs.

These results implicate the *NQO1* message as having a crucial role in the maintenance of the CSC and transformed phenotype of NSCLC. In addition these results support our previous proclamation that therapies focused on reduction of *NQO1* may serve useful in clinic. The application of RNAi technology to reduce expression in patients may provide increased survival and better prognosis through the lessening of the pro-tumor effects demonstrated by the expression of *NQO1*.

Supplemental Figures

Supplemental Figure 2

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A

C-NQO1 2 Query 61 AGCGCCCCGGACTGCACCAGAGCCATGGTCGGCAGAAGAGCACTGATCGTACTGGCTCAC 120
(Clone 1) Sbjct 85 AGCGCCCCGGACTGCACCAGAGCCATGGTCGGCAGAAGAGCACTGATC-----AC 134

B

10 nt
deletion

C

C-NQO1 2 Query 61 AGCGCCCCGGACTGCACCAGAGCCATGGTCGGCAGAAGAGCACTGATCGTACTGGCTCAC 120
(Clone 2) Sbjct 87 AGCGCCCCGGACTGCACCAGAGCCATGG-----CAGAAGAGCACTGATC-----AC 132

D

17 nt
deletion

Supplemental Figure 2. Sequencing validation of C-NQO1 2 CRISPR-Cas9 mediated knockout of NQO1. In **A**, BLAST sequence alignment of C-NQO1 2 (subject) demonstrating a 10 nucleotide deletion, in comparison to the control (query), in one NQO1 allele. In **B**, sequencing histogram illustrating the 10 nucleotide deletion seen in **A**. In **C**, BLAST sequence alignment of C-NQO1 1 (subject) demonstrating a 17 nucleotide deletion, in comparison to the control (query), in the second NQO1 allele. In **D**, sequencing histogram illustrating the 17 nucleotide deletion seen in **C**.

Materials and Methods

Cell Culture

A549, A549 C-NQO1 1, A549 C-NQO1 2, and H596 cell lines were grown in Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine. Cell lines were cultured at 37° C with 20% oxygen and 5% carbon dioxide prior to spheroid culture. Cells were passaged weekly and supplemented with fresh media.

Western blotting

Protein lysates were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with 5% milk in PBST for 1 hour at room temperature, and then incubated overnight with β -actin (1:5000, Santa Cruz Biotechnology) at 4°C. Blots were washed in PBST and incubated for 1 hour with 1:5000 dilution of goat-antimouse IgG-HRP in 5% milk in PBST. The process was repeated using a 1:5000 dilution of monoclonal *NQO1* antibody (clone A-180, Santa Cruz Biotechnology), SOX2 (1:1000, Cell Signaling), Shh (1:1000, Cell Signaling), Oct-4 (1:1000, Cell Signaling), and Nanog (1:1000, Cell Signaling). Pierce ECL western blotting substrate (Thermo Scientific) was used to visual bands on Hyblot-CL autoradiography film (Denville Scientific)

***NQO1* Activity Assay**

NQO1 enzyme activity was performed as previously described. (144) Briefly, 2×10^7 cells of each cell line were collected. Pellets were solubilized in extraction buffer for 20 min, after which they were centrifuged at 18,000 x g for 20 min at 4° C. Supernatants were collected into eppendorf tubes at stored at -80° C. Samples were then run according to the manufacturers protocol for the *NQO1* activity assay kit from Abcam. Results were read at an absorbance of 440 nm every 20 seconds for 5 minutes utilizing the Synergy-H1 Hybrid microplate reader. Plates were shaken both before and after each reading.

CRISPR-Cas9 knockout

CRISPR targeting sequences toward *NQO1* were generated using the available software from crispr.mit.edu. The sgRNA guide sequences that were designed were: *NQO1*: 5'-CACCGCAGAAGAGCACTGATCGTAC-3' and 5'-AAACGTACGATCAGTGCTCTTCTGC-3'. The lentiCRISPRv2 backbone was digested and dephosphorylated using the BsmBI restriction enzyme and gel purified using the QIAEX II Gel Extraction Kit. (Qiagen) The sgRNA guides were then annealed and then ligated with the purified plasmid. Stbl3 bacteria were then transformed with the plasmid via heat shock and plated on carbenicillin containing agar. Colonies were selected and a mini-prep (Qiagen) was performed. Samples were then cut and run on an agarose gel in order to determine sequence insertion. Promising clones were then sequenced. Clones containing the insert were then maxi-prepped and collected plasmid was used to transfect Phoenix 293T cells. Media containing the lentiviral particles was then collected at 24, 48, and 72 hours.

In order to generate *NQO1* knockout cell lines, the lentivirus-containing media was added to cells at a 1:1 ratio with fresh 10% FBS DMEM. The virus was allowed to incubate with the cells for 24 hours after which they began to undergo selection. Selection was carried out using 2 μ g/ml puromycin over a minimum period of two weeks. Following recovery from selection, the cell lines were placed into limited dilution and single cell clones were chosen for expansion. Selected clones were then evaluated via Western blot for *NQO1* expression and subsequently sequenced to confirm *NQO1* disruption.

Spheroid Formation

Low attachment cell culture plates (Corning) were produced by coating the plates with a 0.2% poly-hema/95% ethanol solution. Plates were incubated at 60° C overnight and allowed to dry. The process was then repeated a second time. Plates were washed twice with milli-Q water immediately prior to use.

Cells were trypsinized and treated with trypsin neutralizing solution (1:1 ratio) prior to being counted using a hemacytometer. 10,000 were then plated in the low attachment 100 mm plates in 0.25% FBS DMEM supplemented with 1% L-glutamine. Cells were allowed to form spheroids over 14 days, at which time they were collected, trypsinized into single cell suspensions, and utilized for future assays

. Drug Treatment Studies

Spheroids were collected after 14 days and trypsinized into single cell suspension. Cells were counted and suspended in DMEM media supplemented with 0.25% FBS and 1% L-glutamine at a concentration of 10,000 cells/ml. 200 μ l of cell suspension was then added to each well. Cells were allowed to attach overnight, and the following day were treated with 0, 2.5, 5, and 10 μ M cisplatin dissolved in DMSO. Stock concentrations of 2.5, 5, and 10 mM cisplatin were diluted 1:1000 in 0.25% FBS DMEM supplemented with 1% L-glutamine and incubated on cells overnight. Each dose was performed in 8 replicates for each dose tested. Twenty-four hours after initial treatment, cell viability was assessed using Cell-Titer Glo (Promega), according to the manufacturer's protocol utilizing the Synergy-H1 Hybrid Reader.

Transient and stable *NQO1* mRNA knockdown assays

The human shRNA-*NQO1* retroviral vector was purchased from Open Biosystems. The stable shRNA knockdown cell line (A549 C-*NQO1* sh*NQO1*) was generated by infecting A549 C-*NQO1* cells with polybrene-supplemented medium obtained from Phoenix packaging cells transfected with the human retrovirus vector targeting *NQO1* as described previously (168). Medium was changed 24 hours after transfection. After 48 hours sh*NQO1* containing cells were isolated by limited dilution in media containing puromycin (2 μ g/mL) and screened for *NQO1* mRNA expression levels by real-time qRT-PCR. For transient *NQO1* knockdown, siRNA-*NQO1* or scramble control siRNA (Santa Cruz Biotechnology) was transiently transfected into HCC1171 cell lines (Lipofectamine 2000, Life technologies) using the Life technologies protocol. Cells were harvested after 48 hours and analyzed for *NQO1* mRNA expression.

Anchorage independent growth assays

A 1.5% SeaPlaque Agarose (SPA) mixture was made by slowly adding SPA to PBS and autoclaving. 0.5% SPA was created by diluting the 1.5% stock SPA 1:3 with culture media. 1 mL of the 0.5% SPA mixture was added to each well of a 6 well plate to create a bottom layer and allowed to solidify at room temperature for 15-20 minutes. Cells were counted and suspended at 750 cells/mL in a separate 0.5% SPA mixture. 2 mL were added to each well on top of the bottom layer and allowed to solidify for 30-45 minutes at room temperature to create a cell layer. A 0.3% SPA mixture was created by diluting the 1.5 % stock SPA 1:5 with culture media. 1 mL of the 0.3% SPA mixture was added to each cell layer and allowed to solidify for 20-30 minutes at room temperature to create a top layer. 250-500 μ L of culture media was added onto the top layer to prevent from drying out. Plates were wrapped in parafilm and placed at 37°C. 250-500 μ L of new culture media was added every week. Plates were imaged after 3 weeks using an Epson V700 photo scanner. The enumeration of colonies present in each dish was quantified using imageJ software.

Future Directions

The expression levels of *NQO1* have long been shown to be elevated in a number of malignancies, in comparison to their normal controls (116). This increased expression has led to the targeting of *NQO1* through the utilization of *NQO1* bioactiveable drugs (170, 174). While the bulk of research has focused on improving *NQO1* targeted therapies, there has been very little done to determine the functional role of *NQO1* in the cancer cell. The work presented here aimed to fill in the void of *NQO1*'s function in malignancies by evaluating a number of tumorigenic properties. We demonstrated that *NQO1* was vital for anchorage independent growth, anoikis resistance, invasion, ROS regulation, tumor growth, and ALDH activity. The alteration of a known CSC marker's activity drove us to investigate *NQO1*'s role as a possible CSC marker.

Our investigations into *NQO1* as a CSC marker led to some quite interesting results. We were able to demonstrate that loss of *NQO1* led to a decrease in spheroid formation, in both primary and serial spheroid cultures. In addition, we show that spheroid cultured A549-shCtr cells have increased drug resistance and proliferation. These results strongly support *NQO1* as a major supporter of the CSC phenotype. Additionally we argue that reduction of *NQO1* in patient tumors may be a useful strategy in eliminating the CSC population.

Finally, we investigated the effect of total loss of *NQO1* through the implementation of the CRISP-Cas9 system. To our surprise, complete loss of *NQO1* did not affect sphere formation, soft agar colony formation or the drug resistance that was observed in our shRNA-*NQO1* knockdown studies (**Chapter 2**). In an effort to determine if *NQO1* mRNA was involved, a transient knock down of *NQO1* mRNA by utilization of RNAi technology was implemented. The transient reduction of *NQO1* mRNA led to a decrease in spheroid formation indicating *NQO1* mRNA may be involved in maintaining the transformed phenotype. These data also present the possibility that *NQO1* mRNA is a contributing factor in our previously published work (**Chapter 2**).

The future focus of this project should center on *NQO1* mRNA and the mechanism by which it is affecting both the transformed phenotype, and the CSC population. It has been shown previously that a number of miRs are capable of altering the CSC population. Additionally, the mechanism by which mRNA functions as a miR sponge has been well documented (272-274). Given that reduction of these purported miR leads to an increase in the CSC phenotype, the hypothesis that *NQO1* mRNA is acting as a miR sponge would align with these observations.

Future efforts will need to be made in order to determine the miRs that are capable of binding to *NQO1* mRNA, and whether or not *NQO1* mRNA can function as a suitable mRNA sponge. Some techniques that may be utilized in this effort include RISC complex immunoprecipitation, HITS-CLIP, PAR-CLIP, and labeling of miRs or mRNA in order to perform immunoprecipitation (275-277). Given the massive effort being put forth into identifying and validating miRs, validating those that associate with *NQO1* mRNA will provide future work with a solid foothold on which to build an understanding

of the mechanism of *NQO1* mRNA regulation of tumorigenesis, as well as the cancer stem cell population. Validation of the role of *NQO1* mRNA in tumorigenesis can ultimately lead to development of clinically relevant drugs that target this mRNA, the miRs it regulates, or downstream pathways that may be driving these tumors.

Finally, investigation of *NQO1* mRNA expression and its inhibition in tumors that have an *NQO1* polymorphism may explain why those tumors lacking functional *NQO1* protein may still be capable of forming tumors. To our knowledge there are no exhaustive investigations into the expression of *NQO1* mRNA in polymorphic tumors, however moderate mRNA expression has been reported in polymorphic cell lines (134, 278). It would be interesting to evaluate the mRNA expression in polymorphic cells and demonstrate that reduction of *NQO1* mRNA results in an attenuated tumorigenicity. In closing, there is an unknown amount of information that has yet to be revealed pertaining to *NQO1*. Previous work on *NQO1* mainly focused on utilizing bioactivateable drugs to specifically target *NQO1* over-expressing tumor cells. The future of *NQO1* research should focus more on *NQO1*'s pro-tumorigenic role, as protein over-expression and increased activity does not appear to be the only means by which *NQO1* promotes tumorigenesis.

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