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MCR RapidIMPACT

Molecular Cancer Research

Evaluation of Tumor Cell–Tumor Microenvironment Component Interactions as Potential Predictors of Patient Response to Napabucasin

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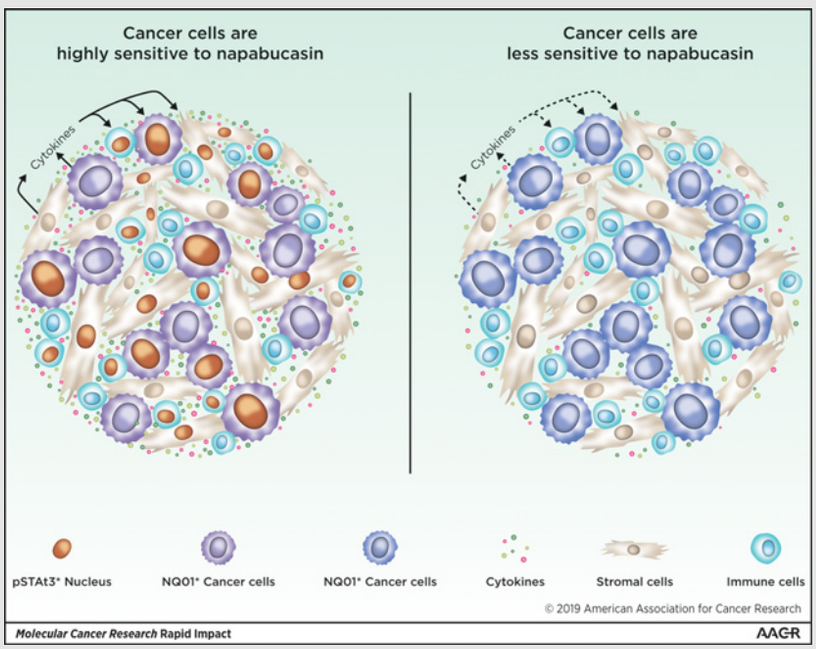
Abstract

Napabucasin is an NAD(P)H:quinone oxidoreductase 1 (NQO1)-bioactivatable small molecule hypothesized to affect multiple oncogenic pathways. In a prespecified, retrospective analysis of the napabucasin phase III CO.23 study, overall survival was longer for napabucasin versus placebo in patients expressing phosphorylated STAT3 (pSTAT3) in tumor cells and cells of the tumor microenvironment (TME). We hypothesized that a connection may exist between NQO1 expression in cancer cells and pSTAT3 in tumor cells and the TME. In 3D spheroid cocultures of cancer cells and cancer-associated fibroblasts, the antitumor activity of napabucasin was NQO1 dependent. The levels of cytokines such as IL6, CXCL10, and GM-CSF were higher in NQO1-positive versus NQO1-deleted cocultures. These differentially secreted cytokines promoted STAT3 phosphorylation in tumor cells and the TME. NQO1-expressing, napabucasin-sensitive tumor cells can modify tumor cells and the TME to promote STAT3 phosphorylation, suggesting that pSTAT3 may be used to identify a subpopulation of patients who would likely respond to napabucasin.



Implications: pSTAT3 is a potential biomarker for patient response to the anticancer drug napabucasin.

Visual Overview: http://mcr.aacrjournals.org/content/molcanres/17/7/1429/F1.large.jpg.

Boston Biomedical Inc., Cambridge, Massachusetts.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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Introduction

Tumor cells often exhibit elevated levels of reactive oxygen species (ROS; ref. 1), which play a role in a variety of cancer processes, including cellular proliferation, survival, metastasis, and angiogenesis (2). ROS mediate these effects by activating intracellular signaling pathways, such as NF-B, MAPK, and PI3K (1). Because ROS can be cytotoxic, tumor cells also upregulate antioxidant pathways (3). One antioxidant enzyme, NAD(P)H:quinone oxidoreductase 1 (NQO1), is overexpressed in a

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number of solid tumors, including breast, lung, and pancreatic (4, 5). Depletion of NQO1 decreases cellular proliferation and growth in a xenograft model of lung adenocarcinoma (4), and in the clinical setting, higher levels of NQO1 have been correlated to a poorer prognosis (5). Therefore, NQO1 has been proposed as a biomarker in a variety of solid tumors (6–9).

However, tumor cells do not exist in isolation, but reside within a microenvironment comprising various cell types [such as cancer-associated fibroblasts (CAF), immune cells, lymphocytes, vascular endothelial cells, and adipose cells] and structural acellular components [extracellular matrix (ECM)], and secreted factors (10). The tumor microenvironment (TME) is increasingly being recognized as a contributor to both tumor progression and treatment resistance (11–13). Consequently, research efforts have begun to focus not only on targeting the tumor, but also the TME (11, 12, 14), as therapies that perturb or alter the function of TME cells or the ECM may improve response and reduce tumor resistance to radiotherapy and chemotherapy (13, 15, 16). Senthebane and colleagues found that esophageal cancer cells increased the expression of ECM collagen and fibronectin proteins in 3D cell–derived ECMs versus normal tissue, and treatment with therapies that inhibit production of collagen and fibronectin proteins of the ECM resulted in decreased esophageal cancer cell survival and reduced chemoresistance (15). Understanding how components of the TME affect tumor progression may provide new targets for cancer therapy.

Napabucasin is an orally administered NQO1-bioactivatable small molecule hypothesized to affect multiple oncogenic pathways. In the phase III CO.23 study, patients with advanced colorectal cancer were randomized to receive either twice daily napabucasin or matching placebo (17). In the total study population, overall survival did not differ between napabucasin (4.4 months) and placebo [4.8 months; HR 1.3; 95% confidence interval (CI), 0.88–1.46]. However, in a prespecified, retrospective analysis of patients expressing phosphorylated STAT3 (pSTAT3) in both cancer cells and the TME, overall survival was longer for napabucasin than for placebo (5.1 vs. 3.0 months; HR 0.41; 95% CI, 0.23–0.73). Given the observations from the CO.23 study (17), we hypothesized that a connection may exist between NQO1 expression in cancer cells and activated STAT3 (as measured by pSTAT3) in tumor cells and the TME. This study sought to further explore the relationship between tumor cells and the TME and assess how tumor cell–TME interactions may affect the response to napabucasin.

Materials and Methods

Cell lines and cultures

FaDu cells, a human epithelial cell line derived from squamous cell carcinoma of the hypopharynx, were purchased from ATCC. Cell line STR validation analysis was performed to verify all cell lines used in this study (Bio-Synthesis, Inc.). To generate *NQO1*-knockout lines, parental cells were transfected with ribonucleoprotein complexes composed of guide RNA (designed with Desktop Genetics Inc.; synthesized by Integrated DNA Technologies, Inc.) and Cas9NLS protein using Lipofectamine CRISPRMAX™ Transfection Reagent (Thermo Fisher Scientific) as per the manufacturer's instructions. See Supplementary Materials and Methods for additional details. Successful gene editing was verified by heteroduplex analysis. Potential *NQO1*-knockout single clones were selected and verified by Western blot analysis.

For a surrogate of the TME, each cancer cell line was cultured in combination with CAFs, specifically hTERT PF179T CAF (ATCC CRL-3290), in 10% DMEM (unless otherwise noted). Frozen human peripheral blood mononuclear cells (PBMC) were purchased from ALLCELLS and cultured in 10% RPMI.

2D and 3D cocultures

2D cocultures were established by seeding tumor cells on top of a 50% confluent fibroblast layer. Tumor cells and CAFs interacted directly for 2–3 days until cocultures became 100% confluent. Supernatant was then collected, aliquoted, and immediately stored at −80°C.

3D spheroid cocultures were established using ultra-low attachment (ULA) U-bottom plates (96 or 384 wells, Thermo Fisher Scientific and Corning) as per the manufacturer's instructions. In brief, exponentially growing cells were harvested and filtered to ensure single-cell suspension. After 10,000 cells were seeded into ULA U-bottom plates at the desired tumor-to-fibroblast ratios, plates were centrifuged at 250 rpm for 5 minutes to facilitate spheroid formation. After formation (2–3 days), spheroids were either treated with napabucasin (Boston Biomedical, Inc.) or collected for immunofluorescence staining. Supernatant was collected, aliquoted, and immediately stored at −80°C.

Antitumor activity of napabucasin

Serial dilutions of napabucasin were added to 3D spheroid cocultures of parental or *NQO1*-knockout cancer cells and fibroblasts. Following 3 hours of exposure to napabucasin, ROS generation was measured using ROS-Glo (Promega). Cell viability was determined using CellTiter-Glo 3D (Promega) per the manufacturer's instructions. Each data point was performed at least in duplicate.

Cytokine secretion assays

Proteome Profiler Human XL Cytokine Array (R&D Systems) was used to identify cytokine secretion in 2D monolayers of FaDu (parental and knockout) cells cultured with CAFs. Cytokine levels in 3D cocultures of FaDu cells were then quantified using ProcartaPlex Assay (Thermo Fisher Scientific) using Luminex technology.

Measurement of pSTAT3

Fibroblast cultures were serum-starved and stimulated for 20 minutes with 100 ng/mL of recombinant CCL2, CCL5, IL6, CXCL1, CXCL8, CXCL10, CXCL11, CXCL12, granulocyte-macrophage colony-stimulating factor (GM-CSF), or soluble vascular cell adhesion molecule 1 (BioLegend). The ability of these recombinant proteins to induce phosphorylation of STAT3 was determined via Western blotting with pSTAT3 (Tyr705) (D3A7) XP rabbit mAb (Cell Signaling Technologies; diluted 1:1,000).

PBMC cultures were serum-starved and stimulated for 20 minutes with 100 ng/mL of each of the aforementioned recombinant proteins. Cells were then fixed, permeabilized, and stained with CD3-PerCP (BioLegend) and pSTAT3-PE antibody (BioLegend) followed by flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher Scientific) to identify pSTAT3-positive cells. Isotype control antibodies (BioLegend) were used to validate true staining.

To examine STAT3 phosphorylation in 3D spheroid cocultures, 48 spheroids at the desired tumor-to-fibroblast ratios

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were collected, fixed, and sectioned for pSTAT3 immunofluorescence staining. Fluorescent spheroid images were analyzed and quantified using CellProfiler (Broad Institute, Cambridge, MA). See Supplementary Materials and Methods for additional details.

Results

Effects of NQO1 on napabucasin-mediated antitumor activity and the TME in 3D spheroid models

To assess the role of tumor cell–CAF interactions on the response to napabucasin, a 3D coculture system of cancer cells and CAFs was employed. In the 3D spheroid model, the viability of FaDu-NQO1–positive cancer cells cultured in the presence of fibroblasts declined with increasing concentrations of napabucasin. In contrast, the viability of NQO1-deleted (FaDu-NQO1^{CR}) cancer cells was not affected by napabucasin (Fig. 1A). In FaDu-NQO1-positive cancer cells, administration of napabucasin was associated with an approximately 6-fold increase in ROS levels. In contrast, ROS levels in FaDu-NQO1^{CR} cancer cells did not increase following treatment with napabucasin (Fig. 1B). Collectively, these data suggest that the antitumor activity of napabucasin is dependent on the generation of ROS and NQO1, with NQO1-positive cancer cells being highly sensitive to napabucasin and NQO1^{CR} cancer cells being comparatively resistant. Given the observation from the CO.23 study that overall survival was longer for napabucasin than for placebo in the subgroup of patients expressing pSTAT3 in both cancer cells and in the cellular component of the TME (17), we hypothesized that a connection may exist between NQO1 expression in cancer cells and pSTAT3 level in tumor cells and the TME. Cytokine arrays were used to measure secreted factors. The levels of 10 cytokines, particularly IL6, CXCL10, and GM-CSF, differed in the supernatants of 2D monolayer FaDu-NQO1-positive versus FaDu-NQO1^{CR} cancer cell cocultures (Fig. 1C). This observation was validated in the 3D spheroid coculture system, where the amount of IL6, CXCL10, and GM-CSF secreted was higher in FaDu-NQO1–positive versus FaDu-NQO1^{CR} cancer cell cocultures (Fig. 1D).

Induction of STAT3 phosphorylation in cells of the TME

We next examined the effects of the cytokines found to be differentially secreted in NQO1-positive versus NQO1^{CR} tumor cell cocultures on phosphorylation of STAT3 in TME cell types. Of the 10 differentially secreted cytokines, only IL6 was found to induce phosphorylation of STAT3 in CAFs cultured in 2D monolayers (Fig. 2A). The immune compartment is an important component of the TME, and pSTAT3 expression may denote the presence of immunosuppressive cells, such as myeloid-derived suppressor cells (18). In human PBMC cultures, IL6 was found to promote STAT3 phosphorylation in both lymphocytes and monocytes; CXCL10 was found to promote STAT3 phosphorylation in CD3-negative lymphocytes; and GM-CSF was found to promote STAT3 phosphorylation in monocytes (Fig. 2B and C). These data suggest that NQO1-positive cancer cells can increase the secretion of factors that are able to promote STAT3 phosphorylation in multiple cell types found in the TME.

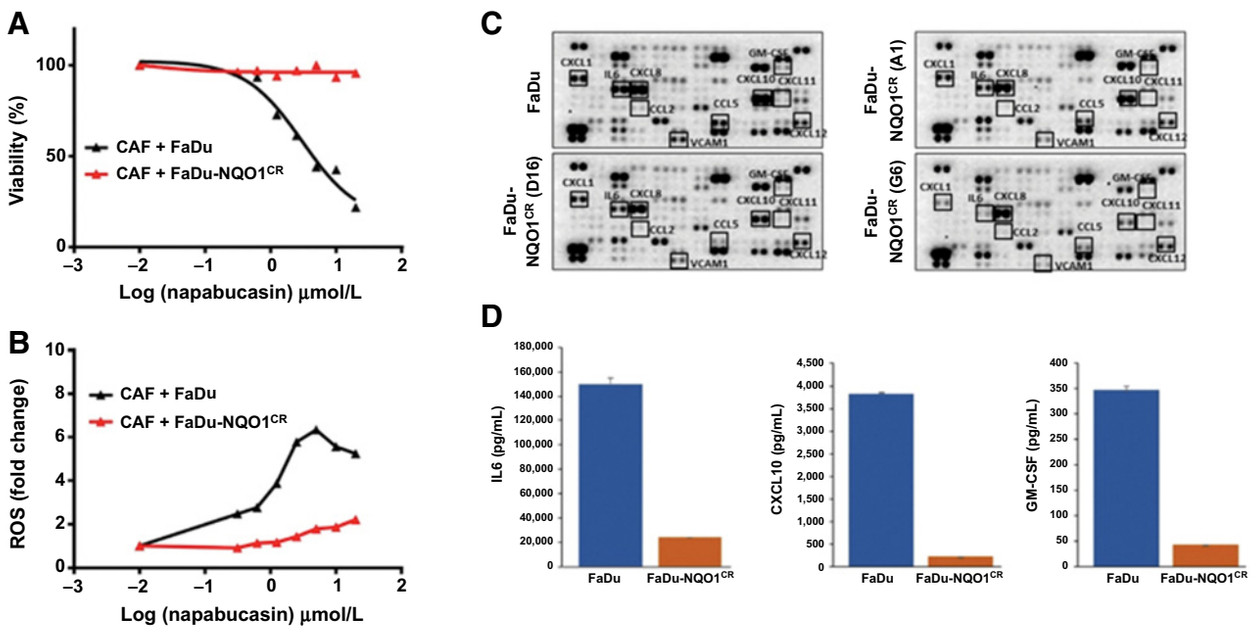

Figure 1.

NQO1 dependency of napabucasin-mediated antitumor activity and the TME in coculture models. **A,** Cell viability assay in 3D spheroid cocultures of tumor cells and CAFs treated with napabucasin. **B,** ROS generation assay in 3D spheroid cocultures of tumor cells and CAFs treated with napabucasin. **C,** Unbiased screen for cytokines differentially secreted in 2D monolayer cocultures of FaDu or FaDu-NQO1^{CR} cancer cells and fibroblasts by proteome profiler human XL cytokine array. Three independent NQO1^{CR} clones (A1, D16, and G6) were compared with parental FaDu cells. Cytokines that depended on tumor expression of NQO1 are denoted by a black box. **D,** IL6, CXCL10, and GM-CSF levels in the supernatant of 3D spheroid cocultures of FaDu or FaDu-NQO1^{CR} cancer cells and CAFs were quantified by ProcartaPlex assay.

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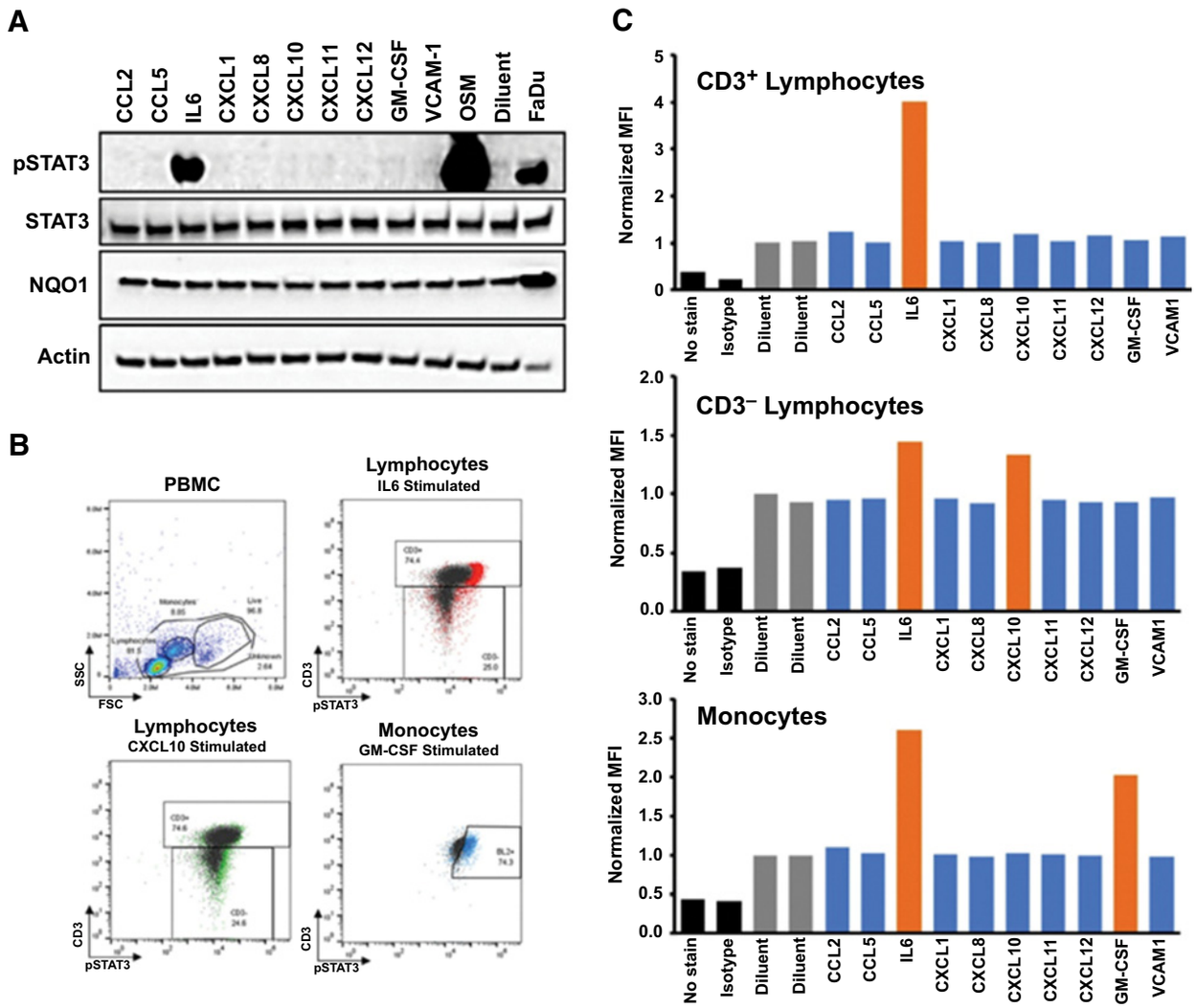

Figure 2.

Differentially secreted factors can promote STAT3 phosphorylation in cell types found in the TME. **A,** IL6 can induce STAT3 phosphorylation in CAFs. **B,** Human PBMCs were costained with CD3 and pSTAT3 and analyzed by flow cytometry. Distinct monocyte and lymphocyte subpopulations in human PBMC cultures were identified in forward versus side scatter plots and confirmed by CD3 expression. Lymphocytes were further divided into CD3-positive (T cells) and CD3-negative (B cells) subsets. The following pSTAT3-stained representative images are shown: IL6-stimulated (red) lymphocytes overlaid with nonstimulated (gray) lymphocytes, CXCL10-stimulated (green) lymphocytes overlaid with nonstimulated (gray) lymphocytes, and GM-CSF–stimulated (blue) monocytes overlaid with nonstimulated (gray) monocytes. **C,** Quantitative results from pSTAT3-stained human PBMCs. *Y* axis indicates the mean fluorescence intensity (MFI) normalized to nonstimulated controls (diluent).

To directly evaluate the effect of tumor cell expression of NQO1 on pSTAT3 in cells of the TME, 3D spheroids were stained with NQO1 and vimentin antibodies to distinguish between tumor cells and CAFs, respectively; the level of nuclear pSTAT3 staining in each cell was then quantified. Representative images from FaDu-NQO1–positive/CAF cocultures and FaDu-NQO1^{CR}/CAF cocultures are shown in Fig. 3A. Upon quantification of multiple spheroid images, including more than 700 CAFs, the presence of NQO1 in cancer cells was found to promote phosphorylation of STAT3 in CAFs. The signal intensities of pSTAT3 in both cancer cells and CAFs differed significantly between FaDu-NQO1–positive/CAF cocultures and FaDu-NQO1^{CR}/CAF cocultures ($P < 0.0001$; Fig. 3B). As expected, parental FaDu cells expressed higher levels of NQO1 than CAFs. FaDu-NQO1^{CR} cocultures exhibited minimal staining of NQO1 (Fig. 3C). Vimentin was expressed at similar levels in fibroblasts from FaDu-NQO1–positive and FaDu-NQO1^{CR} cocultures, with low levels of expression in FaDu cancer cells (Fig. 3D). Together, these data indicate that NQO1-positive and NQO1^{CR} tumor cells can differentially modify the level of STAT3 phosphorylation in both tumor cells and cells of the TME.

Discussion

The elevated levels of ROS present in cancer cells promote increased expression of NQO1 (3). Although NQO1 expression in

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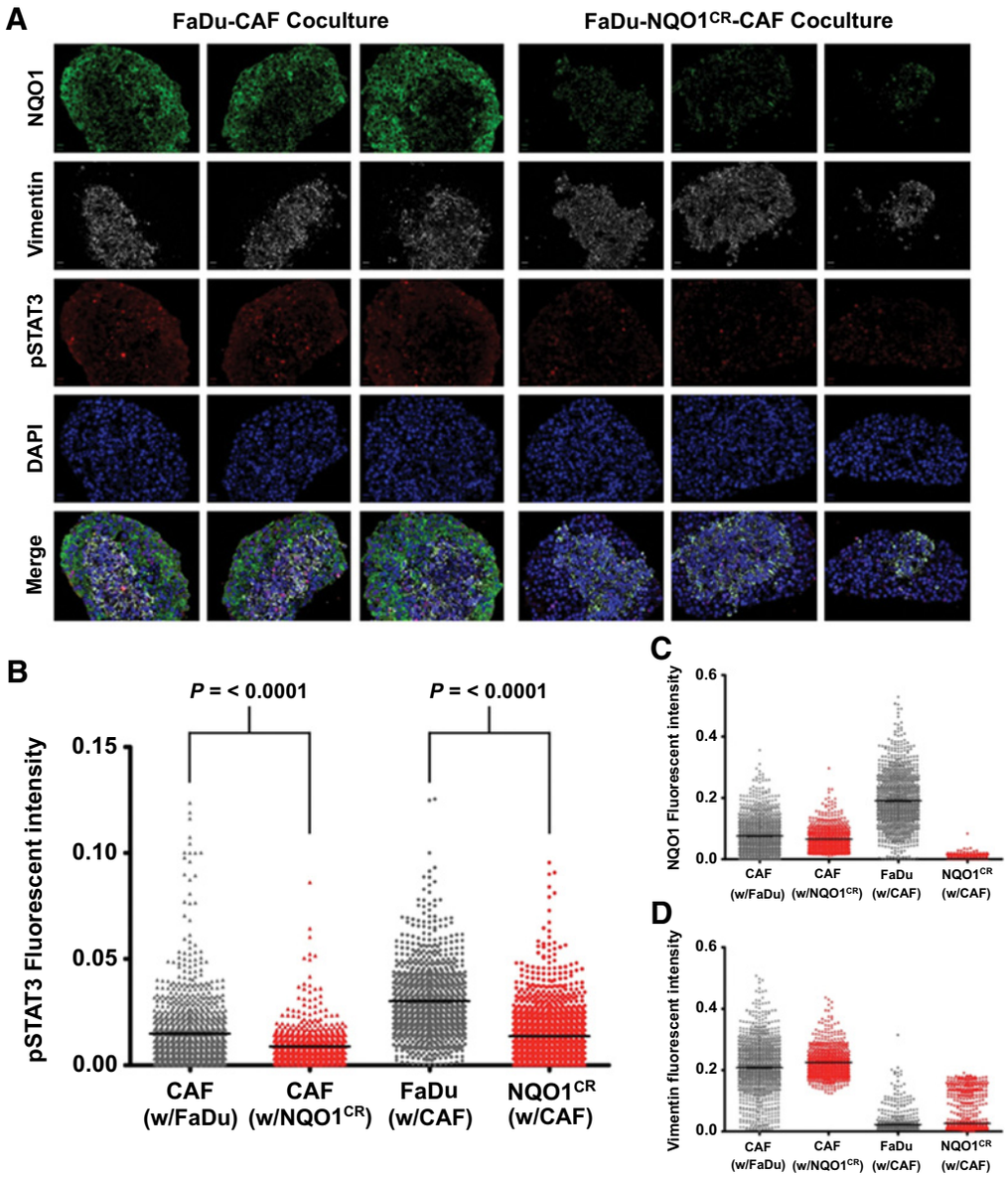

Figure 3.

NQO1-positive tumor cells in 3D spheroid cocultures promote STAT3 phosphorylation in the TME. **A,** Representative immunofluorescence images of FaDu or FaDu-NQO1^{CR} tumor cells cultured with CAFs. The signal intensities of pSTAT3 (**B**), NQO1 (**C**), and vimentin (**D**) in each cell in the spheroid were plotted. Tumor cells and CAFs were plotted separately.

cancer cells can promote drug resistance and contribute to a cancer stem cell phenotype (4, 19), elevated NQO1 levels also provide a therapeutic target for NQO1-bioactivatable compounds. As an NQO1-bioactivatable compound, napabucasin requires the activity of NQO1 to generate high levels of ROS and kill cancer cells. Notably, napabucasin-mediated ROS generation not only functions as a mediator of cancer cell death, but also affects cell signaling pathways (20).

Compared with nontransformed cells, redox balance in cancer cells is altered to allow for increased cellular proliferation and tumor development (1, 21). To compensate for the higher levels of ROS and to prevent ROS-induced cytotoxicity, cancer cells also express higher levels of antioxidant proteins (1, 21). One such antioxidant enzyme, catalase, has been shown to inhibit the activity of another NQO1-bioactivatable compound, β-lapachone (22). The balance between the levels of NQO1-generated ROS and antioxidants therefore can impact the threshold of napabucasin-generated ROS necessary for precipitating cancer cell death. Even though NQO1 is required for the antitumor activity of napabucasin, the complex nature of redox balance in cancer cells precludes the exclusive use of NQO1 as a possible biomarker of napabucasin activity.

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Our data also indicate that NQO1-positive cancer cells, which are sensitive to napabucasin, interact with cells of the TME in a manner that is distinct from NQO1-negative cells, which are less sensitive to napabucasin. Although the exact mechanism is unclear, expression of NQO1 in tumor cells correlates with increased secretion of multiple cytokines, including IL6, CXCL10, and GM-CSF—cytokines, which promote an immunosuppressive microenvironment. These secreted factors can act on the tumor cells themselves, as well as cells of the TME. In conclusion, our observations suggest that napabucasin-sensitive cancer cells can modify tumor cells and cells of the TME to promote STAT3 phosphorylation and that pSTAT3 level may serve as a biomarker of tumor redox balance, which preliminary evidence suggests is correlated to the antitumor activity of napabucasin (17).

Disclosure of Potential Conflicts of Interest

Y. Li is a research associate at Boston Biomedical Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A.-Y. Chang, H. Iguchi, H.A. Rogoff
Development of methodology: A.-Y. Chang, E. Hsu, J. Patel, Y. Li, H.A. Rogoff
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.-Y. Chang, E. Hsu, Y. Li, M. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.-Y. Chang, E. Hsu, J. Patel, M. Zhang, H.A. Rogoff
Writing, review, and/or revision of the manuscript: A.-Y. Chang, E. Hsu, J. Patel, M. Zhang, H.A. Rogoff
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.-Y. Chang, E. Hsu, Y. Li, H.A. Rogoff
Study supervision: A.-Y. Chang, H.A. Rogoff

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