

Cul3 overexpression depletes Nrf2 in breast cancer and is associated with sensitivity to carcinogens, to oxidative stress, and to chemotherapy

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Abstract

Nrf2 is the key transcription factor for cytoprotective gene programs. Nrf2 is normally maintained at very low concentrations by proteasomal degradation, through its interaction with the adapter protein Keap1 and the Cul3 E3 ligase. Increased Nrf2 concentration resulting from loss of function Keap1 mutations has been described in chemoresistant non-small cell lung cancer. Previous studies in breast cancer showed low levels of some Nrf2-regulated detoxification genes, but the mechanism has not been systematically examined. We found that half of the breast cancer cell lines examined have decreased concentration of Nrf2 compared with normal mammary epithelial cell lines, associated with variable but detectable levels in Keap1 levels, and consistently increased Cul3 mRNA and protein. Immunohistochemistry showed that 7 of 10 breast cancer specimens examined also have low Nrf2 levels and increased Cul3. Keap1 protein levels are variable. We found no C23Y mutation in Keap1 of any of the cell lines. Using siRNA, we silenced Cul3 in MCF-7 breast cancer cells, and microarray analysis reveals the induction of GCL, NQO1, AKR1C1, UGDH, and TXN by at least 2-fold. The Nrf2-regulated ABCC1 drug transporter was also found to be increased.

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These Cul3-silenced MCF7 cells are highly resistant to oxidative stress induced by H₂O₂, to the carcinogen benzo(a)pyrene, and to both Doxorubicin and Paclitaxel. This high Cul3/low Nrf2 signature may be key to cellular sensitivity to both chemical carcinogenic stimuli as well as to cytotoxicity of commonly used chemotherapeutic drugs in established breast cancers. [Mol Cancer Ther 2009;8(8):2432–40]

Introduction

The importance of cellular defense in carcinogenesis has been documented in a variety of models and contexts. Studies of cancer-prone DNA repair syndromes and mutations in susceptibility genes have clearly established that these changes occur at an increased rate when cells have deficiencies in protective mechanisms against genotoxic stress (1–6). DNA damage, mutations, and tumorigenesis are increased in mouse model with deficiencies in antioxidant and detoxifying systems (7, 8). Another consequence of deficient genoprotective mechanisms is the increased sensitivity to reactive oxygen species and carcinogens, as lethal doses are lower in cells and tissues of affected individuals (9). *In vitro* and *in vivo* studies of normal and cancer cells have shown that inhibition of antioxidant or detoxifying systems sensitize cells to a wide array of chemicals, while accelerating the removal of DNA lesions or increasing the redox and detoxifying potentials has opposite effects (10–13). Further adding to the notion that antioxidant and detoxification systems play a significant role in carcinogenesis, many dietary and synthetic putative chemopreventive agents act by inducing antioxidant and detoxifying enzyme (14, 15).

The comprehensive system that detoxifies and discards environmental and endogenous toxins, mutagens, and potential carcinogens is composed of multiple antioxidant and detoxifying enzymes that are classified as phase I and phase II detoxifying enzymes and transporters participating, respectively, in the oxidation, conjugation, and elimination of genotoxins (16–19). Nrf2 is a master transcriptional regulator of antioxidant and detoxifying enzymes, which binds to the *cis*-acting antioxidant response element present in the promoters of multiple phase II detoxifying genes in a heterodimeric complex with a small Maf protein (16–18). The Nrf2-regulated battery of carcinogen-detoxifying enzymes includes glutathione *S*-transferase (GST), γ -glutamylcysteine synthetase (GCL), NAD(P)H:quinone oxidoreductase1 (NQO1), and multiple UDP:glucuronosyl transferases. In a mouse Nrf2 knockout model, constitutive hepatic and gastric activities of GST and NQO1 are greatly reduced; the inducibility of others is lost; the chemopreventive effect of a dithiolethione, which increase the cellular redox potential via stimulation of glutathione synthesis, is

lost; and benzo(a)pyrene (BaP)-induced tumorigenesis is increased (9, 20). Another mechanism of cellular protection against toxic chemicals involves efflux transporters in the ABC gene family, and Nrf2 regulation has been shown for ABCC1 (21, 22).

The ubiquitin/proteasome degradation system plays an important role in regulating stability and cellular localization of Nrf2, and therefore its activity. Under basal redox conditions, the redox-sensitive protein Keap1 tightly binds to Nrf2 and anchors it in the cytoplasm through another interaction with filamentous actin. Keap1 serves as an essential adaptor protein for the Cullin 3 ubiquitin E3 ligase (Cul3), which then specifically targets Nrf2 for degradation by the ubiquitin-proteasome pathway and, under physiologic conditions, maintains normal cells with a very low level of Nrf2 protein (21–23). The relative amount of Keap1 and Cul3, i.e., degree of saturation of Keap1 by Cul3, is determinant of Nrf2 levels. Upon oxidative stress, Keap1 undergoes conformational changes that disrupt its interaction with Nrf2. Nrf2 is no longer degraded and free to translocate in the nucleus. Recent work has also identified Keap1's control of Nrf2 function via nuclear exporting of Nrf2 (23–26). The Cullins constitute an evolutionarily conserved family of proteins. Each individual Cullin can assemble into multiple E3 ligases by interacting with a protein motif present in multiple proteins (27, 28). There is a growing body of evidence suggesting that Cullin-dependent ubiquitin ligases play important roles in breast carcinogenesis. For example, gene amplification of Cul4A has been reported in 16% of primary breast cancers (29). The candidate tumor suppressor RhoBTB2 binds Cul3 and is a likely substrate-specific adaptor protein for Cul3, in a manner analogous to Keap1, and it functions by targeting oncogenic proteins for proteosomal degradation (30).

It has long been noted that the protein levels of some antioxidant and detoxifying enzymes are decreased in several malignancies, including breast cancer. The data from our laboratory and others showed decreases in detoxifying enzymes, such as GST isoenzymes, in breast cancers (31, 32). However, the mechanism(s) responsible for these findings are not fully understood. Interestingly, a recent study has uncovered a link between Nrf2 levels and resistance to chemotherapy drugs in established tumors. This study identifies mutations resulting in loss of function of Keap1, resulting in Nrf2 activation of antioxidant, xenobiotic metabolism, drug efflux pumps, and in intrinsic chemoresistance in non-small cell lung tumors (33, 34).

Breast cancer is generally more chemosensitive than non-small cell lung cancer, and the Nrf2-Keap1-Cul3 axis has not been systematically examined in breast cancer in relation to cancer therapeutics. In fact, genome-wide sequencing of a number of breast cancer clinical specimens also has identified a Keap1 mutation as a rare event, and a subsequent functional analysis of the specific NH₂ terminus point mutation that was identified was shown in cells to disrupt the Nrf2-Keap1-Cul3 association responsible for Nrf2 ubiquitination and degradation (35, 36). We studied Nrf2 and its regulation in breast cancer, and in both tissue samples and in

breast cancer cell lines, we found a frequent signature of low Nrf2 related to high Cul3 levels. Keap 1 levels vary, but not consistent with the changes observed in Nrf2. Mechanistic studies show increased Cul3-mediated degradation is responsible for decreased Nrf2, which results in modulation of cellular detoxifying systems and chemosensitivity to cytotoxic drugs used in breast cancer therapy. Some breast cancers and some breast cancer cell lines have higher Nrf2 levels, and this was not associated with the previously identified Keap1 C23Y mutation. Other factors, or mutations at other Keap1 sites, are clearly also at play.

Materials and Methods

Chemicals and Antibodies

MG132 was purchased from Sigma Aldrich from the National Cancer Institute Chemical Repository, Midwest Research Institute. The antibodies against Nrf2, Keap1, and Cul3 were from Santa Cruz Biotechnology Co. The rabbit polyclonal antibodies against the GSTA1/2, GSTP1, GCL, and NQO1 were generously provided by Dr. John D. Hayes (University of Dundee, Scotland, United Kingdom). The rabbit antibody against the AKR1C was a kind gift from Dr. Andrew Stolz (University of Southern California, Los Angeles, CA).

Clinical Samples

Matched clinical breast cancer and the surrounding normal mammary tissues were obtained from 10 patients undergoing surgery with written informed consent and approval by the Research Ethics Board of the Jewish General Hospital.

Cell Culture

The breast cancer cell lines were obtained from American Type Culture Collection and maintained in RPMI 1640 with 10% fetal bovine serum. The normal human mammary epithelial cells (HMEC) were purchased from Clonetics and maintained in mammary epithelial growth medium, supplemented with 2 mL bovine pituitary extracts, 0.5 mL hEGF, 0.5 mL Hydrocortisone, 0.5 mL GA-1000, and 0.5 mL Insulin (MEGM Bullet kit; Cambrex).

Western Blotting and Immunohistochemistry

Thirty micrograms of nuclear extract or 60 µg of cytoplasmic extract were resolved by SDS-PAGE, transferred onto nitrocellulose membranes. The membranes were blocked with 5% fat-free milk solution and then sequentially incubated with indicated primary antibodies (1:1,000) and the appropriate peroxidase-conjugated secondary antibody (1: 5,000). The results were documented on X-ray films with electrochemiluminescence detection. Membranes were reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. For low abundant proteins, which here included both Nrf2 and Keap1, cells were transfected with either Nrf2 or Keap1, to provide lysates with overexpressed protein. They were used to confirm the proper identification of the target protein. Tumor biopsies were fixed with neutral buffered formaldehyde, and embedded in paraffin. Nrf2, Keap1, and Cul3 were detected on microsections using Vectastain ABC kit according to the manufacturer's instructions

Table 1. List of primers used in RT-PCR

Gene	Primer pairs (sequences 5'→3')	
	Forward	Reverse
Nrf2	TCACCATCTCAGGGGGAG	CAACATACTGACACTCCAATGC
Cul-3	AGAGCGGAAAAGGAGAAGTCGTAGA	CTCAAAGTCACCCGCAATAGTT
Keap1	CAGCCAAGGTCCCTGAGT	GTGTAGCTGAAGGTGCGGT
γGCS	AGAGAAGGGGGAAAGGACAA	GTGAACCCAGGACAGCCTAA
GSTP1	CCCTACACCGTGGTCTATTTC	GAGGCTTTGAGTGAGCCCT
NQO1	TGAAGGACCCTGCGAACTTTC	GAACACTCGCTCAAACCAGC
AKR1C1	CAATTCCCATCGACCAGAGT	TACTTGAGCCCTGGCTTGTT
UGDH	GATGGGCTCCACAATGAACT	TGGGCAACAGTGAGACTCTG
Thioredoxin	CAACCCTTCTTTCATTCCTCT	CACCCACCTTTGTCCCTTCT

(Vector Laboratories). Counterstaining with H&E staining was applied for examination of tissue morphology and determination of Nrf2 and Cul3 subcellular localization.

Generation of Stable Cul3 Knockdown Cells

Two RNA interfering sequences were selected according to the human Cul3 gene (Gene bank accession number BC039598). Sequences spanning the nucleotides 2044-2062 and 2526-2548 were cloned into a pMSCV-puro-based retroviral vector (pSUPER-retro) according to the manufacturer's protocol (Oligoengine). Empty and interfering Cul3 sequences containing pSUPER-retro were transfected into the Phoenix amphotrophic packaging cell line to produce infectious retroviral particles. Forty-eight hours posttransfection, the viral supernatants were collected, filtered on 0.45-μm filters, and incubated with polybrene (8 μg/mL) for 48 h with the MCF-7 breast cancer cell line. Polyclonal populations stably expressing the different Cul3-directed short interfering RNAs (siRNA) or no siRNA were selected with 2 μg/mL puromycin for 2 wk. Both targeting sequences efficiently knocked down Cul3 in the MCF-7 breast cancer cell line as determined by Western blotting. Control MCF-7 cells infected with an empty vector or the Cul3-targeting sequences are referred to as PSR, Cul3.1, and Cul3.2, respectively.

Gene Expression Profiling of Control versus Cul3-silenced MCF-7 Cells

Control (PSR) and Cul3-silenced cells (Cul3.1 and Cul3.2) were harvested at 70% to 80% confluence. Chips "Whole Human Genome Oligo Microarray kit" (Agilent Technologies) were probed with total RNA isolated with the RNeasy mini kit (Qiagen). The chip hybridization and slide scanning were done according to Agilent protocols. Reverse transcription-PCR (RT-PCR) and Western blotting analyses were combined to validate the expression of selected genes that had a 2-fold or more variation in expression.

Gene Expression Analysis by RT-PCR

Total RNA was isolated from the different breast cancer and HME cells with the Trizol reagent, according to the manufacturer's protocol (Invitrogen). The mRNA amplification was done with the One Step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. Each RT-PCR reaction contained 20 ng of total RNA, 250 μmol/L deoxynucleotide triphosphates, 50 pmols of each primer, and 10 unit of enzyme in a final volume of 25 μL. The RT-PCR pro-

file was 50°C for 30 min, 95°C for 15 min, followed by 32 to 35 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 1 min, and a final 10-min extension at 72°C. RT-PCR products were analyzed on 1.0% agarose gels. Sequences of the primers used are listed in Table 1.

hABCC1 Relative Quantification

cDNA was generated from RNA using random primers and Superscript II reverse transcriptase (Invitrogen). ABCC1 gene expression was determined using Power SYBR green master mix (Applied Biosystems) with the following primer set: 5'-GGGACTCAGGAGCACACGAA-3' and 5'-AAATGCCAGGGCTCCAT-3'. Primers were designed using the Primer Express 3.0 (Applied Biosystems). GAPDH was used as the endogenous control and was determined using a VIC-labeled Taqman probe and Taqman Fast Master Mix (Applied Biosystems). QPCR was done on the 7500 Fast Real-time PCR system using standard parameters and analyzed using relative quantification with untransfected MCF7 as the calibrator.

Sequence Analysis of Keap 1

RT-PCR was done using a one-step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. Briefly, total RNA from HMEC, MCF7, MDA 231, MDA 468, Hs578T, and ZR75 cells with low Nrf2 protein, and from MDA 435, BT20, and T47D, which have higher Nrf2 levels, was isolated using Trizol (Invitrogen), and 100 ng were synthesized into cDNA and amplified by PCR using Keap 1-specific primers (forward primer 5'-CAGCCAAGGTCCCTGAGT-3'; reverse primer 5'-GTGTAGCTGAAGGTGCGGT-3'; Invitrogen). The total cycle number for the reaction was 35 cycles. The reaction yielded a product ~200 nucleotides long, which corresponded to the region containing the Keap1 C23Y mutation. Following the reaction, the products were separated on a 1.2% agarose gel and were gel extracted using the QIAquick Gel Extraction kit (Qiagen). To detect the mutation, the RT-PCR products were sequenced (Bio S&T).

Cytotoxicity Assays

The MCF-7 wild-type, MCF-7 PSR control, and the Cul3-silenced cells were seeded in 96-well plates at a density of 2,000 cells per well. After 24 h, the cells were treated with BaP, H₂O₂, Doxorubicin, or Paclitaxel at the indicated concentrations. Cell proliferation was evaluated 5 d postaddition of

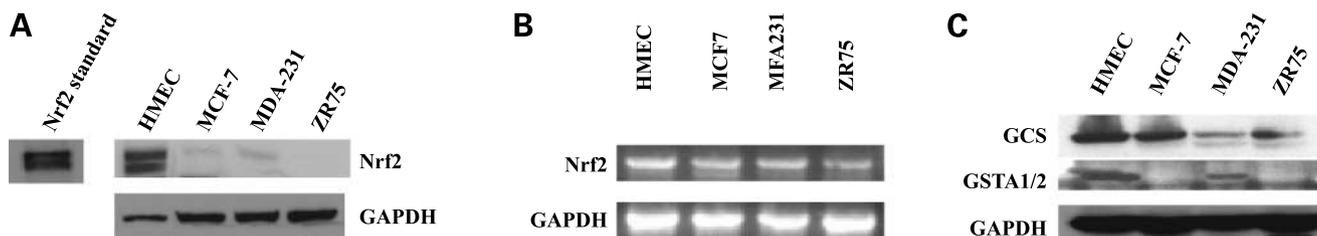


Figure 1. Nrf2 is depleted in breast cancer cell lines. Nrf2 protein and RNA levels in breast cancer cell lines were compared with HMECs. **A**, Western blotting for nuclear Nrf2 in MCF-7, MDA-231, and ZR-75 breast cancer cell lines. **B**, Nrf2 mRNA expression detected by RT-PCR on total RNA extracts. **C**, Western blotting for basal expression of GCL and GSTA1/2 in MCF-7, MDA-231, and ZR-75 breast cancer cell lines. GAPDH was used as a loading control.

BaP or H₂O₂. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine sensitivity to BaP, whereas the sulforodamine assay was used to determine sensitivity to H₂O₂. The absorbance was determined at 570 nm with a microplate reader (Bio-Rad, model 450). Cell survival is expressed as percentage of cell growth in comparison with the solvent-treated cells. The unpaired Student's *t* test was used to compare between the treated and the untreated cells (*, *P* < 0.05).

Results

Nrf2 Is Frequently Depleted in Breast Cancer Biopsies and Breast Cancer Cell Lines by a Proteasome-Dependent Mechanism

In light of a report demonstrating increased Nrf2 levels in intrinsically chemoresistance lung cancer cells (33, 34), we used Western blot analysis to examine a panel of breast cancer cell lines. We found significantly decreased Nrf2 protein in 7 of 10 cell lines examined, when compared with two separate normal mammary epithelial cell lines (HMEC). This was observed in cell lines that are both hormone receptor positive and negative, as exemplified by three tumor cell lines (MCF-7, MDA231, ZR-75) in Fig. 1A. To determine the mechanism of diminished Nrf2, we used RT-PCR and found only a minor reduction in the mRNA levels of Nrf2 in these three breast cancer cell lines when compared with HMEC (Fig. 1B), supporting the notion that a posttransla-

tional mechanism is involved in Nrf2 protein depletion in these breast cancer cells. To confirm that proteasomal degradation was responsible for the observed reduction in Nrf2 in these cells, MCF-7 cells were treated with the proteasome inhibitor MG132, and we observed a strong increase in Nrf2 protein level (data not shown). Decreased Nrf2 protein correlates with diminished activity, as the Nrf2-inducible expression of the antioxidant enzymes GCL and GSTA1/2 is strongly reduced (Fig. 1C). Keap 1 levels varied among all of the cell lines and compared with the NMEC, but without a direct relation to the level of Nrf2. Figure 2A shows the level of Keap 1 protein in the same three cell lines. The cells with the highest Keap 1 levels (MCF-7) are not the cells with the lowest Nrf2 protein level (ZR-75). RT-PCR measurement of Keap1 mRNA shows no dramatic difference among the cell lines and compared with NMECs. Cul3 protein levels were found to be significantly elevated in the same three cell lines with low Nrf2 protein levels, Fig. 2A. All three cell lines also had high Cul3 mRNA concentration (Fig. 2B). In the other three cell lines (MDA 435, BT20, and T47D), the Nrf2 protein levels was higher, although Cul3 levels are comparable with the rest of the cell lines tested, and Keap1 concentration was not different or related to Nrf2 levels. In the three "high Nrf2" cell lines, we looked for either lower levels of Keap1 compared with the other cell lines, or the recently described C23Y Keap1 mutation to explain this phenotype, but neither of these was detected.

In light of the data in breast cancer cell lines, we examined 10 breast cancer tissue samples, obtained from our tumor bank with Research Ethics Board-approved consent. The morphologic diagnosis as well as hormone (ER, PR) and Her2 neu receptor status was recorded. Each sample was also immunostained for Nrf2, Cul3, and Keap1. Seven specimens had low Nrf2 levels when compared with the surrounding tissue, and this was associated with increased Cul3. The remaining three samples had Nrf2 levels similar to surrounding normal tissue. Figure 3 shows representative images, in which the top panel shows immunostaining against Nrf2 protein in malignant (A) versus nonmalignant (B) sections from the breast cancer specimens, and the bottom panel shows staining for Cul3 in the malignant (C) versus nonmalignant (D) sections of tissue. Keap1 protein levels were not significantly different between normal and tumor tissue, although the signal using all of the available antibodies was extremely weak (data not shown). There

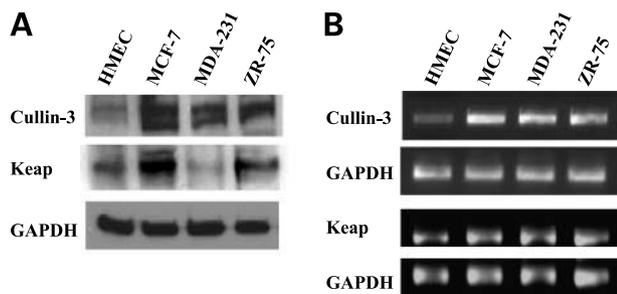


Figure 2. Cul3 is overexpressed, and Keap 1 varies in expression in Nrf2 depleted breast cancer cell lines. Breast cancer cell lines with low Nrf2 levels were assessed for Cul3 and Keap1 protein levels compared with HMECs. **A**, Western blotting for total Cul3 or Keap1 in HMEC and in MCF-7, MDA-231, and ZR-75 breast cancer cell lines. **B**, Cul3 and Keap1 mRNA expression detected by RT-PCR on total RNA extracts from the same cell lines. GAPDH was used as a loading control.

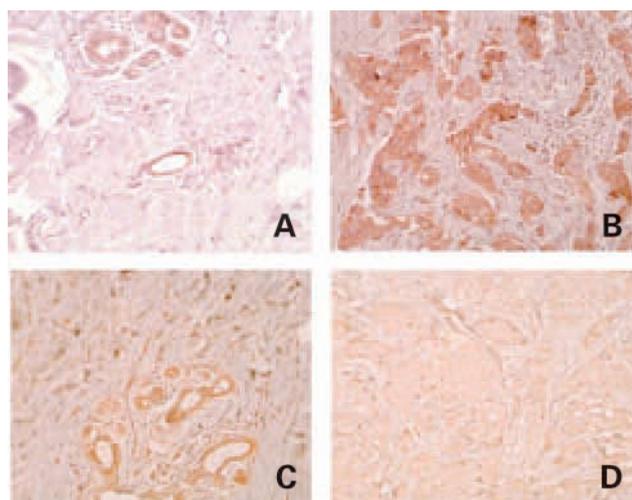


Figure 3. Cul3 is overexpressed in Nrf2-depleted breast cancer tumors. In 7 of 10 samples, breast cancer tissue samples expressing low Nrf2 level and matching surrounding normal mammary tissues were stained by immunohistochemistry for Cul3. Representative photomicrographs of 10 samples. **A**, normal mammary tissue showing Cul3 expression. **B**, breast cancer tissue showing Cul3 overexpression. **C**, normal mammary tissue with the presence of Nrf2, compared with **(D)** cancer cells with significantly lower Nrf2 signal.

was no obvious correlation between the differentiation status or level of expression of the receptors analyzed and either Nrf2 or Cul3 levels.

Cul3 Silencing Induces Nrf2 and Multiple Detoxifying Genes in Breast Cancer Cells

To address the function of Cul3 overexpression in regulating Nrf2 protein stability in breast cancer cells, we designed two short interfering RNAs (siRNACul3.1 and siRNA-Cul3.2) and constructed two stable retrovirus-transduced MCF-7 polyclonal cell populations, both of which showed silencing of Cul3 protein expression. In these two Cul3-silenced cell populations (MCF7-Cul3.1 and MCF7-Cul3.2), the Nrf2 protein level is constitutively higher (Fig. 4A). These results show that Cul3 overexpression contributes to Nrf2 protein loss in breast cancer cells.

The effect of Nrf2 restoration on its transcriptional activity was determined by microarray on two independent cell cultures from each of Cul3-silenced cell population. The patterns of gene expression from both Cul3-silenced cell populations were very similar, indicating that changes in gene expression levels are specific to Cul3 silencing and not to off-target effects (Table 2). Phase I and phase II enzymes that have significantly altered expression levels are listed in the table, including aldo-keto reductase family 1, member C1 (AKR1C1), GCL, and UDP-glucose dehydrogenase (UGDH). Up-regulated mRNA levels of AKR1C1, GCL, and UGDH in the Cul3-silenced cells were confirmed by RT-PCR (Fig. 4B). Western blotting confirmed elevated protein expression of AKR1C1 and GCL in the Cul3-silenced cells (Fig. 4C); UGDH protein levels were not assessed due to a lack of reliable antibodies.

The resistance observed in the Cul3-silenced MCF7 cells cannot be attributed to an increase in the multidrug transporter Pgp (ABCB1), as the protein level is not increased in these cells and Pgp gene regulation is not associated with Nrf2 regulation (data not shown). Because MRP (ABCC1) can be regulated by Nrf2, we compared the relative basal expression of ABCC1 between MCF7, the vector-transfected PSR, and the Cul3 knockdown cells using quantitative PCR. Knockdown of Cul3 resulted in a 3-fold increase in ABCC1 mRNA expression when compared with either the untransfected or vector-transfected MCF7 cells (Fig. 4C). There is no difference between the control and MCF7 cells, and the SiRNA-silenced cells have statistically significantly increased hABCC RNA ($P < 0.01$).

Cul3 Silencing in Breast Cancer Cells Increases Resistance to H₂O₂, to BaP Cytotoxicity, and to Anticancer Drug Cytotoxicity

We sought to determine the significance of increase in phase I and phase II enzymes levels in the context of genotoxic and cytotoxic stresses, and examined Cul3.1 cells in cell survival studies. Cul3-silenced cells are resistant to oxidative stress due to H₂O₂ relative to the wild-type and control cells (IC₅₀ for Cul3-knockdown cells versus wild-type and control cells were 3.62 μ mol/L versus 1.17 μ mol/L and 1.21 μ mol/L, respectively; Fig. 5A), which is statistically significant for the SiRNA-silenced cells ($P < 0.01$).

BaP can be modified into active metabolites such as BaP epoxide, which in turn forms BaP-DNA adducts, causes oxidative stress and cell death at high doses (37–40). The inhibitory concentrations of BaP are lower for wild-type MCF7 and control cells than in Cul3-silenced cells (Fig. 5B). The IC₅₀ values for Cul3-knockdown cells versus wild-type and control cells were 514.36 nmol/L versus 93.23 nmol/L and 117.75 nmol/L, respectively, which is statistically significant for the SiRNA silenced cells ($P < 0.01$).

Examination of two important cytotoxic drugs used to treat breast cancer showed a significant impact of Nrf2 increase resulting from Cul3-knockdown. The IC₅₀ values for Doxorubicin in the Cul3-knockdown cells versus wild-type and control cells were 0.5 μ mol/L versus 0.0025 μ mol/L (Fig. 5C), and for Paclitaxel, they were 2.25 nmol/L versus 0.75 nmol/L (Fig. 5D), which is also statistically significant for the SiRNA silenced cells ($P < 0.01$).

Together, these results show that Nrf2 depletion associated with Cul3 overexpression in breast cancer in the presence of Keap1 protein, and the resulting limitation on a variety of cell detoxification mechanisms, may contribute to increased sensitivity to oxidative stress, carcinogens, and even to cytotoxic therapeutic agents in established cancer cells.

Discussion

In this study, we found that Nrf2 protein levels are very low in a significant number of breast tumors and cell lines compared with normal mammary epithelial cells examined, which is caused by the overexpression of the E3 ubiquitin ligase Cul3, at both the protein and the mRNA levels. Although Keap1 protein does vary among the cell lines we

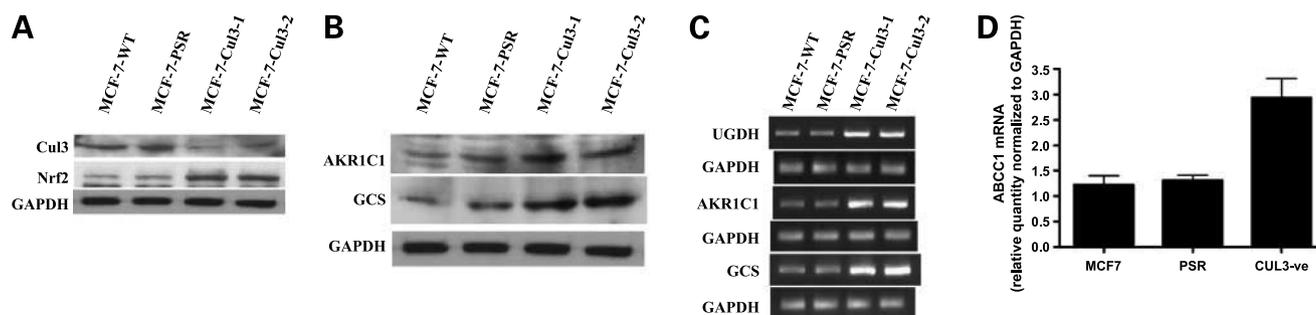


Figure 4. Nrf2 and phase I/II enzymes are induced in Cul3-silenced breast cancer cells. Cul3 was stably silenced in MCF-7 cells using two different siRNA retroviral constructs (Cul3.1 and Cul3.2) to two different polyclonal populations as described in Materials and Methods. The expression of Cul3, Nrf2, and the enzymes AKR1C1, GCL, and UGDH was determined in parental MCF7 cells (wild-type), MCF7 transduced with an empty retroviral construct (control), and the two siRNA Cul3 (Cul3.1 and Cul3.2). **A**, Western blotting for total Cul3 and Nrf2. **B**, Western blotting for nuclear AKR1C1 and GCL; GAPDH was used as a loading control. **C**, AKR1C1, GCL, and UGDH mRNA expression detected by RT-PCR on total RNA extracts. **D**, relative quantification of hABCC1 transporter using Quantitative PCR.

studied, it alone was not directly related to Nrf2 levels, and Keap1 mRNA levels were not significantly altered. On the other hand, Cul3 mRNA and protein is significantly elevated in all of the low Nrf2 cells. The relationship between these proteins is critical, dependent on specific binding functions that result in ubiquitination the Nrf2, and perhaps Keap1 as well, and the potential for mutations in either Keap1 or Nrf2 (33, 41). Our data suggest that Cul3 is elevated in many breast cancer cells, and they suggest that in the presence of adequate levels of functional Keap1, which is found in all the cells we tested, this Cul3 elevation results in greater Nrf2 depletion. We are examining further the dynamic relationship of these three genes and their protein products in cancer cells. SiRNA silencing of Cul3 results in increased Nrf2 protein and concomitant induction of several antioxidant response element-inducible phase I/II enzymes including GCL and AKR1C1, and increased expression of the efflux pump ABCC1. UGDH mRNA is also increased, and it has also been related to proliferation in breast cancer cells (42), although the Cul3-silenced cells seem to grow slower than the controls. Consistent with the induction of antioxidant and detoxifying enzymes, Cul3-silenced cells show increased resistance to oxidative stress induced by hydrogen peroxide, serum starvation, and the carcinogen BaP. Cul3 silencing in these breast

cancer cells also results in significant resistance to both Doxorubicin and Paclitaxel, which may at least in part be due to increased ABCC1. The elucidation of a mechanism responsible for depleting Nrf2 and impairing antioxidant and detoxifying capabilities in breast cancer may have implications in understanding mammary carcinogenesis as well as for breast cancer treatment.

Mammalian cells constantly face genotoxic stresses caused by oxidants, xenobiotics, and other potential carcinogens. The concerted actions of phase I/II enzymes form an array of detoxification mechanisms. It has been shown that oxidative stress is strongly increased in breast cancer (37) and that BaP-induced DNA adducts accumulate in breast cancer cells to much higher levels than do normal mammary epithelial cells (38), which could result from compromised antioxidant and detoxifying capabilities. BaP is a complete carcinogen that we have previously used to generate partially transformed human breast epithelial cell lines (39), and which has been suspected to have a potential role in human carcinogenesis, including breast cancer. Moreover, it has been shown that the production of reactive oxygen metabolites is higher in breast cancer tissues irrespective of clinical stage (40), raising the possibility that deregulation of redox metabolism occurs early in mammary carcinogenesis. The finding that expression levels and activities of

Table 2. Phase I and phase II enzymes up-regulated in Cul3-silenced MCF-7 cells

Genebank accession no.	Detoxifying enzyme	Fold increase	Function
NM_014862	Aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2)	3.69 ± 0.27	Phase I
NM_000104	Cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1)	4.02 ± 0.45	Phase I
NM_001353	Aldo-keto reductase family 1, member C1 (AKR1C1)	4.29 ± 0.791	Phase II
NM_003774	Udp-N-acetyl- α -D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 4 (GalNAc-T4)	4.15 ± 0.97	Phase II
NM_199127	γ -Glutamyltransferase-like 4 (GGTL4)	2.67 ± 0.50	Phase II
NM_003312	Thiosulfate sulfurtransferase (rhodanese; TST)	2.08 ± 0.55	Phase II
NM_003329	Thioredoxin (TXN)	1.63 ± 0.10	Phase II
NM_003359	UGDH	7.70 ± 0.43	Phase II

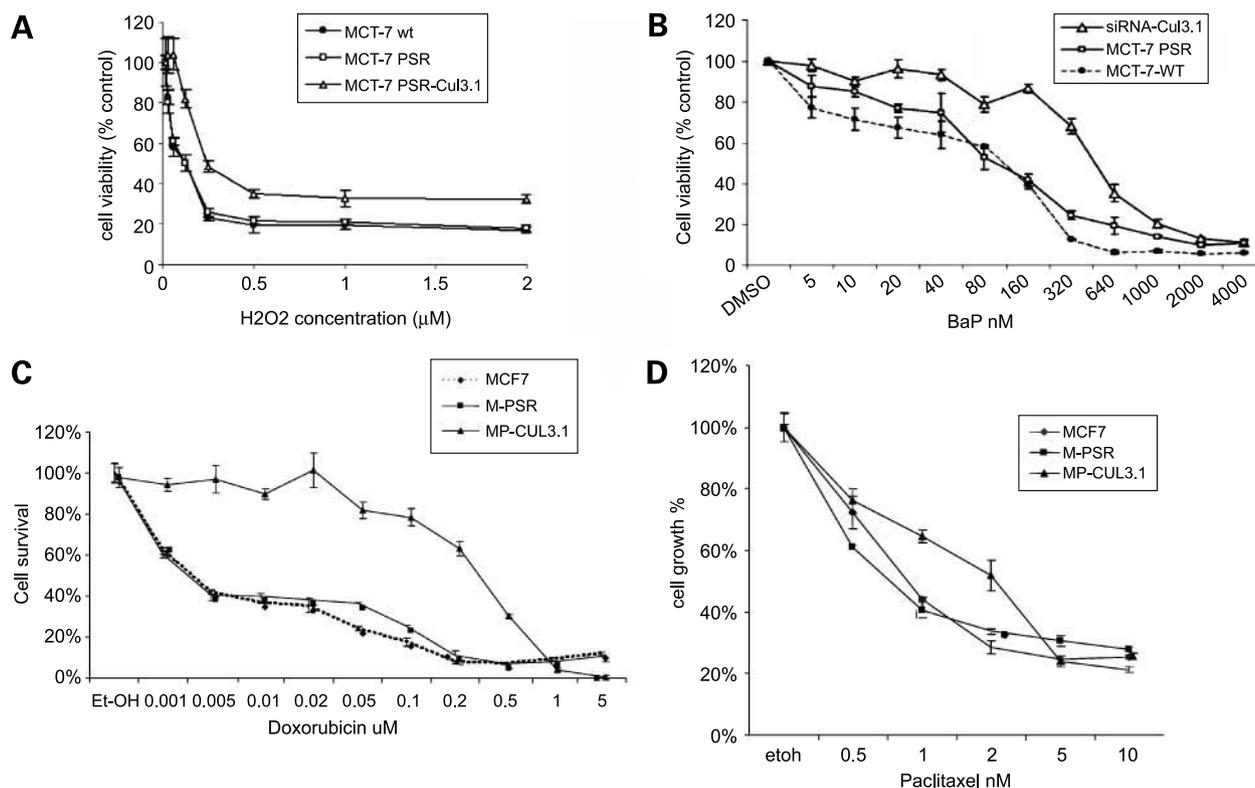


Figure 5. Cul3-silenced MCF-7 cells are resistant to H₂O₂- and serum starvation-induced cytotoxicity. Cul3-silenced cells were compared with wild-type and control cells to examine the cytotoxicity of (A) H₂O₂, (B) BaP, (C) Doxorubicin, and (D) Paclitaxel. Cell survival curves with data from at least three separate experiments are shown; *points*, mean; *bars*, SD.

enzymes of the glutathione metabolism are reduced in breast tumors has shed some light on underlying mechanisms of increased oxidation in breast cancer and has provided targets for therapeutic intervention (31). The role of individual genes of the antioxidant and detoxifying pathways in breast cancer susceptibility and treatment is not certain; a more global search may be more effective in the identification of biomarkers of cell sensitivity. We have shown that the master regulator Nrf2 is frequently decreased in breast tumors as well as in cancer cell lines and that this may limit the activity, or inducibility, of several mechanisms of cellular detoxification.

A further potential link between Nrf2 depletion and decreased antioxidant capability in breast cancer is data on the hereditary breast cancer-associated gene BRCA1, which has been shown to enhance Nrf2 activity and to increase the resistance to oxidative stress (43). BRCA1-deficient cells are hypersensitive to oxidation caused by ionizing radiation and hydrogen peroxide (44). Whether the association between breast cancer risk and BRCA1 mutations depends in some part on loss of Nrf2 activation remains to be elucidated; however, inactivation of BRCA1 is one mechanism for negative Nrf2 regulation (44). We show that Cul3 overexpression is an efficient mechanism for Nrf2 inactivation in breast cancer cell lines, and it would be so regardless of a mechanism inducing Nrf2 such as BRCA1. The interaction

of Cul3 and BRCA1 in regulating Nrf2 protein levels has not been examined; however, it is clear that BRCA1 increases Nrf2 activity and xenobiotic stress-inducible gene expression, and is required for Nrf2-independent induction of p450s CYP1A1 and CYP1B1 (45).

Although the Cul3/Keap1/Nrf2 interaction could be key to cellular sensitivity to chemical carcinogenesis, the implications for the therapy of established cancers has become more evident with the recent demonstration that intrinsically chemotherapy-resistant non-small cell lung cancer frequently harbors mutations in Keap1 protein that interfere with Nrf2 turnover. The resulting increase in Nrf2, as a result of loss of function of Keap1-Cul3 interaction, is associated with significant resistance to clinically relevant chemotherapeutic agents (33, 34). A specific Keap1 mutation was identified as a rare event in genome-wide sequencing of clinical breast cancer specimens, and this mutation, at position C23Y, was also shown in a cellular system to also disrupt Nrf2 degradation resulting from Keap1 interactions (35, 36). We did not find this mutation in Keap1 in the breast cancer cell lines, which have higher Nrf2 protein levels, although other Keap1 mutations are certainly possible and are the subject of other work. Although Keap1 protein does vary among the cell lines we studied, it seems to be present in adequate abundance, and not to contain the C23Y loss of function mutation, such that the level of

Cul3 becomes determinant of Nrf2 activation or degradation. We found that both Cul3 protein and mRNA levels are increased in the low Nrf2 cells. The relationship between these proteins is critical, dependent on specific binding functions that result in ubiquitination the Nrf2, and perhaps Keap1 as well, and the potential for mutations in either Keap1 or Nrf2 (46). Our data further show that Cul3-silenced cells are >20-fold more resistant to the cytotoxic effect of doxorubicin, suggesting that Cul3 induction and/or Nrf2 depletion may be interesting targets for chemosensitization. Finally, it is striking to find increased Cul3 protein, possibly due to increased gene transcription, given the increased mRNA. In view of the previously reported observation of amplification of Cul4A, a related Cullin gene, in breast cancer, more work is anticipated to fully develop an understanding Cul3 overexpression in breast cancers (32).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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