

# The Nuclear Matrix Protein, NRP/B, Enhances Nrf2-Mediated Oxidative Stress Responses in Breast Cancer Cells

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## Abstract

**The transcription factor NF-E2-related factor 2 (Nrf2) translocates into the nucleus and activates phase II genes encoding detoxification enzymes and antioxidant proteins, resulting in the protection of cells from oxidative insults. However, the involvement of Nrf2-mediated oxidative stress responses in breast cancer cells is largely unknown. Notably, during our study of the Nrf2 pathway in breast cancer cells, we observed that the nuclear matrix protein NRP/B was expressed and colocalized with Nrf2 in these cells, suggesting that NRP/B is involved in Nrf2-mediated oxidative stress responses. The expression level of NRP/B was variable in different breast cancer cells and breast cancer tissues, and was found to be localized in the nucleus. NRP/B expression was increased after exposure to the oxidative stress agent, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), particularly in the highly aggressive MDA-MB-231 breast cancer cells. Association of NRP/B with Nrf2 *in vitro* and *in vivo* was observed in MDA-MB-231 breast cancer cells, and this association was up-regulated upon exposure to H<sub>2</sub>O<sub>2</sub>, but not to sodium nitroprusside, SIN-1, and DETA-NO. NRP/B also enhanced Nrf2-mediated NAD(P)H:quinine oxidoreductase 1 promoter activity. Thus, this study reveals that NRP/B enhances oxidative stress responses in breast cancer cells via the Nrf2 pathway, identifying a novel role of nuclear matrix protein(s) in oxidative stress responses. [Cancer Res 2007;67(18):8596–604]**

## Introduction

Cellular metabolism is required for the biological functions of cells and their generated toxic by-products, which are physiologically removed by detoxification or clearance systems. Impairment of this system results in the intracellular accumulation of toxic-free radicals and abnormal proteins, which cause oxidative stress-induced damage to cells, leading to lipid peroxidation, DNA oxidation, and carbonylation, which then disrupt the function of lipids, DNA, and proteins. These processes result in pathologic consequences, and subsequently to cancer progression (1–3). Coordinated induction of cytoprotective genes enables the cells to neutralize reactive molecules and restore cellular redox homeostasis (4, 5). The antioxidant response element (ARE) that transcriptionally regulates phase II genes encoding detoxification enzymes and antioxidant

proteins, such as NAD(P)H:quinine oxidoreductase 1 (NQO1), has an important role in cytoprotection (4–6).

NF-E2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that binds to the ARE promoter sequence, leading to the coordinated up-regulation of ARE-driven detoxifying and antioxidant genes (7, 8). In response to oxidative stress factor(s), the kelch-like ECH-associated protein 1 (Keap1) releases Nrf2, resulting in translocation and activation of downstream targets that boost cellular detoxification processes and antioxidant potential (7, 9–12). The Nrf2 system plays a critical role in protecting a variety of tissues from a wide array of toxic insults (carcinogens, reactive oxygen species, diesel exhaust, inflammation, calcium disturbance, UV light, and cigarette smoke; ref. 13).

Nuclear matrix proteins are reported to be involved in many biological processes such as transcription, cell cycle, and primary transcription processing, and are also linked to intermediate filaments of the cytoskeleton (14–17). Furthermore, nuclear matrix proteins have been implicated in malignant transformation (18, 19), such as alterations in DNA ploidy, DNA content, nuclear shape, and proliferative states (20). The nuclear matrix protein, termed NRP/B (ENC-1), is expressed abundantly in the nervous system (21, 22), and its expression has been observed in various brain cell lines, human brain tumors (23, 24), and hairy cell leukemia (25). NRP/B and Keap1 are members of the kelch-related family of actin-binding proteins, possessing sequences homologous to the *Drosophila* kelch protein (22, 26–28). The kelch proteins have two characteristic motifs. The first motif consists of ~115 amino acids termed the BTB domain (Broad-complex, Tramtrack, Bric-a-brac; refs. 29, 30). The other motif consists of ~50 amino acids repeated in tandem and is termed the kelch repeats (30). Although Keap1 was shown to be a transcriptional repressor of Nrf2, NRP/B involvement in oxidative stress responses remains obscure.

Oxidative stress induces the translocation of Nrf2 into the nucleus, resulting in activation of phase II detoxifying enzyme genes and antioxidant proteins. The cytoplasmic-nuclear passage of Nrf2 occurs through nuclear matrix networks. Surprisingly, while studying the involvement of Nrf2 in oxidative stress responses in breast cancer cells, we found that NRP/B was expressed and colocalized with Nrf2 in these cell lines. These results led us to assess the possible involvement of NRP/B in oxidative stress responses. This study reveals for the first time that NRP/B is involved in oxidative stress responses via the Nrf2 pathway in breast cancer cells. These findings further suggest a novel avenue of nuclear matrix protein(s) involvement in Nrf2-mediated oxidative stress responses in these cancer cells.

## Materials and Methods

**Chemicals.** Diethylenetriamine nitric/oxide adduct (DETA-NO), sodium nitroferricyanide (III) dehydrate [sodium nitroprusside (SNP)],

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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3-morpholinysydnone hydrochloride (SIN-1), and hydrogen peroxide ( $H_2O_2$ ) were obtained from Sigma. Catalase (human erythrocytes) was purchased from Calbiochem.

**Cell cultures.** Cell culture conditions were undertaken as recommended by the American Type Cell Collection. MCF-7 cells were cultured in MEM medium with 2 mmol/L L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate, and supplemented with 0.01 mg/mL bovine insulin and 10% fetal bovine serum (FBS). MDA-MB-231, T-47D, and 293T cells were grown in RPMI 1640 with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate, and supplemented with 0.2 units/mL bovine insulin and 10% FBS. HCT116 cells were cultured in McCoy's 5a medium with 1.5 mmol/L L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate, and 10% FBS.

**RNA isolation.** RNA isolation was done using TRIzol reagent (Invitrogen). The procedure was carried out according to the manufacturer's instructions. One milliliter of TRIzol reagent was added, followed by the addition of 0.2 mL of chloroform after 15 min. Samples were vigorously inverted by hand for 15 s, and centrifuged at  $12,000 \times g$  for 15 min at  $4^\circ C$ . The aqueous phase was transferred to a fresh tube and 0.5 mL of isopropyl alcohol was added. Samples were centrifuged at  $12,000 \times g$  for 10 min at  $4^\circ C$ . The pellets were washed once with 75% ethanol, dissolved in RNase-free water, and incubated for 10 min at  $60^\circ C$ . RNA concentration was measured and stored at  $-80^\circ C$ .

**Northern blot analysis.** In brief, 20  $\mu g$  of RNA purified from cells were loaded and run on 1% gel. The RNA gel was transferred onto Hybond-N+

(Amersham) in  $20\times$  SSC overnight. Membranes were cross-linked and prehybridized in prehybridization solution [ $5\times$  saline-sodium phosphate-EDTA (SSPE), 2% SDS,  $5\times$  Denhardt's reagent, 100  $\mu g/mL$  denatured Salmon testes DNA] at  $65^\circ C$  for 6 h. Purified PCR-generated NRP/B (BTB domain) served as a probe and was labeled with  $\alpha^{32}P$  using the NEblot kit (New England Biolabs). The membranes were incubated with hybridization solution containing the NRP/B-labeled probe at  $65^\circ C$  overnight, and then washed once in washing solution ( $0.5\times$  SSPE, 1% SDS) for 20 min, and twice in washing solution ( $0.1\times$  SSPE, 1% SDS) for 20 min each. Membranes were then exposed to X-ray film at  $-80^\circ C$ .

**DNA transfection.** 293T cells and transfected with DNA constructs using LipofectAMINE. For the MDA-MB-231 cells, transfections were done using a Nucleofector device (Amaxa) per the manufacturer's instructions. Briefly, cells ( $1 \times 10^6$ ) were harvested and resuspended with 100  $\mu L$  of the provided Nucleofector solution. The cell suspension was mixed with 1.5  $\mu g$  of DNA constructs and then transferred to a certified cuvette. The cuvette was inserted into the Nucleofector device and the appropriate program (X-13) was selected and started. Immediately, the cells were replated and cultured for 24 h followed by cell extraction.

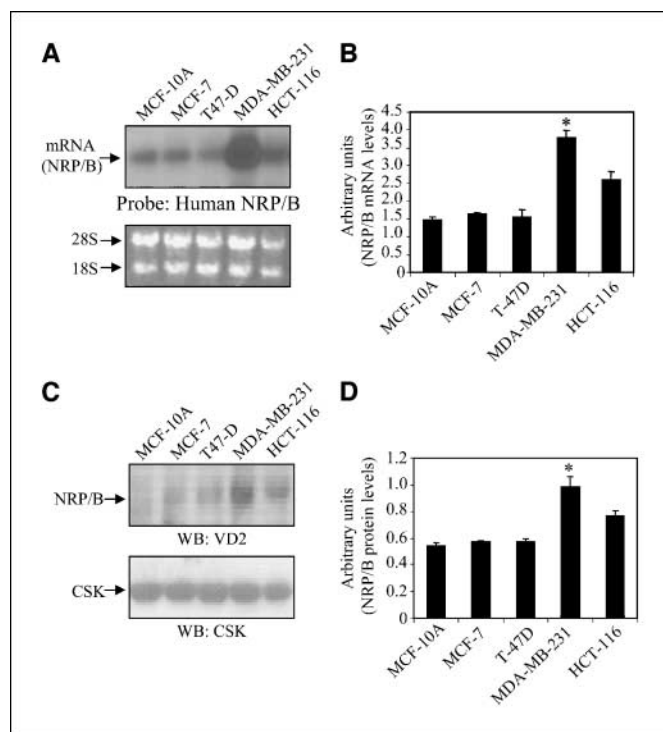
**Immunocytochemistry.** Cell cultures were fixed with 4% paraformaldehyde for 15 min. The cultures were treated with 0.1% Triton X-100 in PBS for 30 min. After three washes, cells were treated with 10% goat serum in PBS for 2 h and incubated with anti-NRP/B (VD2) and/or anti-Nrf2 antibodies for 1 h at room temperature. Following three washes, cells were incubated with FITC-conjugated IgG and/or Tris-conjugated IgG antibodies for 1 h. Cells were then washed, and the slides were mounted and images were taken using a confocal microscope (Zeiss LSM 510 Meta). To examine the *in vitro* colocalization of NRP/B and Nrf2, pEGFP-Nrf2 and Myc-NRP/B constructs were transfected into 293T cells. After 24 h of transfection, the cells were fixed and blocked with 3% skim milk and stained with anti-Myc antibody. Images were then taken using the confocal microscope described above.

**Immunoblot analysis.** Cell cultures were washed with PBS and lysed directly on ice with cold lysis buffer [100 mmol/L KCl, 300 mmol/L sucrose, 10 mmol/L Pipes (pH 6.8), 3 mmol/L  $MgCl_2$ , 1.2 mmol/L phenylmethylsulfonyl fluoride, 0.5% Triton X-100, and 1 mmol/L EGTA]. The lysates were transferred to a new tube followed by solubilization for 1 h at  $4^\circ C$ . Total cell lysates were clarified by centrifugation at 12,000 rpm for 20 min at  $4^\circ C$ . The concentration of the cell extracts was determined using a protein assay. Equal amounts of proteins were applied to 10% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. The blots were incubated with anti-NRP/B (VD2), anti-Nrf2, or anti-CSK antibodies. After three washes, the blots were incubated with horseradish peroxidase-IgG antibody.

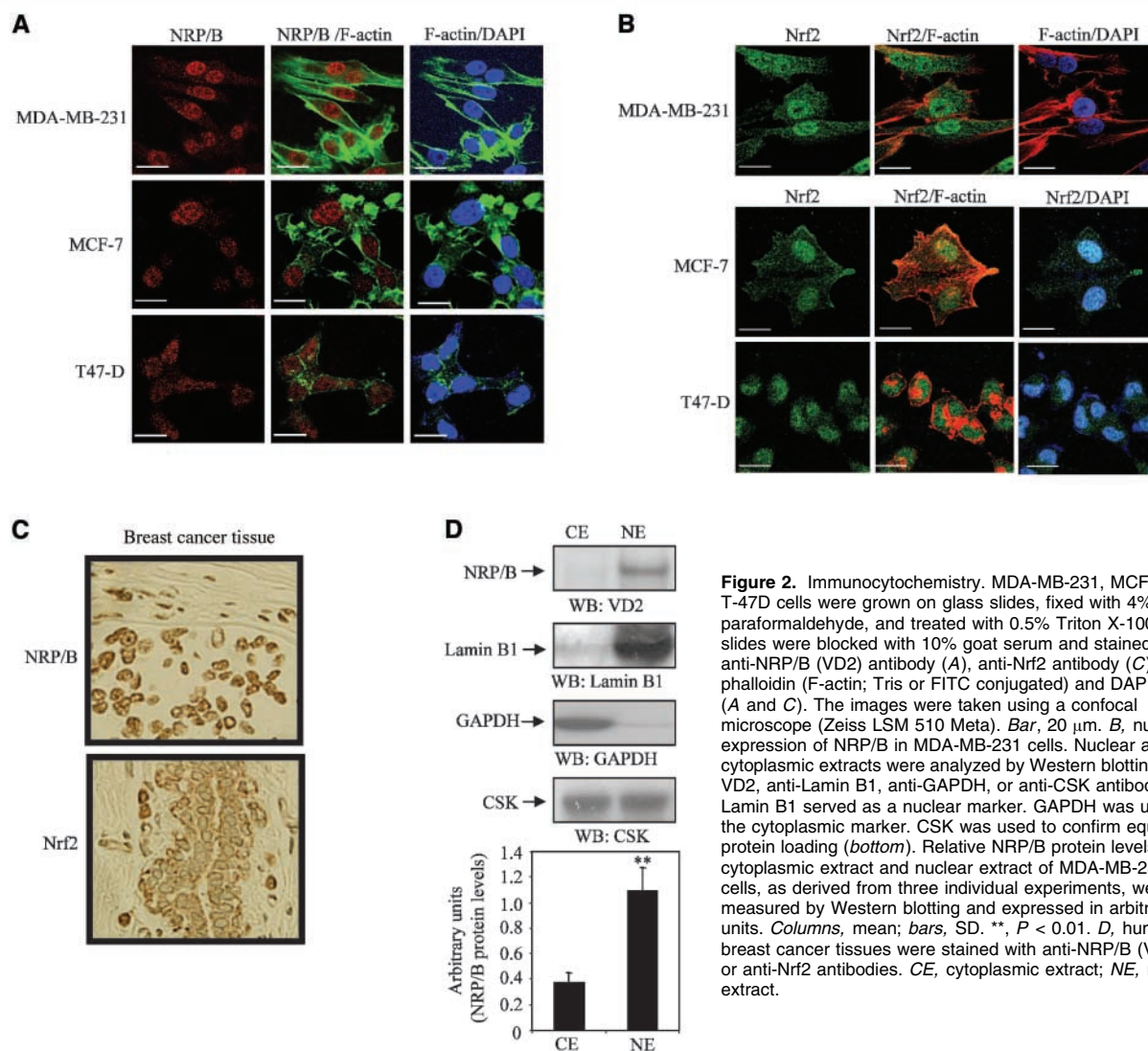
**DNA constructs.** The mammalian expression constructs for human NRP/B were generated by amplifying the human cDNA of NRP/B using the following primers: hNRPB-F4: 5'-AAGCTTTTCAGTCAGTGTGCATGAG-3' and hNRPB-R5: 5'-GATATCTTAAGAAGGCAGATGTTTCCAG-3'. The product was inserted into the pFLAG-CMV4 vector at the HindIII and EcoRV restriction sites. The resulting construct was designated as pFlag-NRP/B. We also generated a construct targeting the expression of NRP/B in the nucleus. The latter construct was generated by PCR using primers as follows: hNRPB-F11 5'-CGTCGACTCAGTCAGTGTGCATGAG-3' and hNRPB-R1 5'-TGC CGCAGAAGGCAGATGTTTCCAG-3'. PCR was digested with *SalI* and *NotI*, and ligated into the pCMV/myc/nuc vector (Clontech). The resultant construct was designated as pMyc-NRP/B. The sequences and the expression of all constructs were verified. pEGFP-Nrf2 was kindly provided by Dr. Laurie Zipper (Georgetown University, Washington, DC).

**Immunoprecipitation.** 293T cells were cotransfected with pFlag-NRP/B, pMyc-NRP/B, or pEGFP-Nrf2. Following 24 h of transfection, the cultures were washed twice with PBS and lysed with cold lysis buffer on ice. After preclearing, the extracts were immunoprecipitated with anti-Flag or anti-Nrf2 antibodies. The precipitates were then blotted and probed with anti-Flag, anti-Nrf2, or anti-Myc antibodies, accordingly. MDA-MB-231 cells were treated with  $H_2O_2$ . Cells were collected, lysed, and subjected to immunoprecipitation with VD2 or anti-Nrf2 antibody. The precipitates were blotted and incubated with VD2 or anti-Nrf2 antibodies.

**Glutathione S-transferase pull-down analysis.** Cells were lysed and then rotated for 30 min at  $4^\circ C$ . To remove the nonspecific binding to



**Figure 1.** Expression and localization of NRP/B in human breast cancer cell lines. *A*, for Northern blot analysis, total RNA was purified from breast cancer cell lines using TRIzol reagent. Ten micrograms of RNA were blotted on a Hybond-N<sup>+</sup> membrane and hybridized with an  $\alpha^{32}P$ -labeled human NRP/B probe. *B*, relative NRP/B mRNA levels derived from three individual experiments were measured by Northern blotting and expressed in arbitrary units. *C*, total cell lysates were prepared from several cell lines, and 100  $\mu g$  samples were blotted on a PVDF membrane. The blot was incubated with anti-NRP/B (VD2) or anti-CSK antibodies. CSK was used to confirm equal protein loading. *WB*, Western blot. *D*, relative NRP/B protein levels derived from three individual experiments were measured by Western blotting and expressed in arbitrary units. Columns, mean; bars, SD. \*,  $P < 0.05$ .



**Figure 2.** Immunocytochemistry. MDA-MB-231, MCF-7, and T-47D cells were grown on glass slides, fixed with 4% paraformaldehyde, and treated with 0.5% Triton X-100. The slides were blocked with 10% goat serum and stained with anti-NRP/B (VD2) antibody (A), anti-Nrf2 antibody (C), phalloidin (F-actin; Tris or FITC conjugated) and DAPI (A and C). The images were taken using a confocal microscope (Zeiss LSM 510 Meta). Bar, 20  $\mu$ m. B, nuclear expression of NRP/B in MDA-MB-231 cells. Nuclear and cytoplasmic extracts were analyzed by Western blotting using VD2, anti-Lamin B1, anti-GAPDH, or anti-CSK antibodies. Lamin B1 served as a nuclear marker. GAPDH was used as the cytoplasmic marker. CSK was used to confirm equal protein loading (bottom). Relative NRP/B protein levels in the cytoplasmic extract and nuclear extract of MDA-MB-231 cells, as derived from three individual experiments, were measured by Western blotting and expressed in arbitrary units. Columns, mean; bars, SD. \*\*,  $P < 0.01$ . D, human breast cancer tissues were stained with anti-NRP/B (VD2) or anti-Nrf2 antibodies. CE, cytoplasmic extract; NE, nuclear extract.

glutathione *S*-transferase (GST), clear protein extracts were incubated with Sepharose beads for 1 h and centrifuged. The supernatants were incubated with GST or GST-NRP/B at room temperature for 1 h. After three washes with lysis buffer, the precipitates were subjected to immunoblotting analysis.

**Reverse transcription-PCR analysis.** Levels of transcripts were semi-quantified by reverse transcription-PCR (RT-PCR). One-step RT-PCR was done based on the manufacturer's instructions using a set of specific primers. Thioredoxin reductase (*TRX*): 5'-GCTCAGGAGGTCTGGCAGCTGCTAAG-3' and 5'-GTGCAAGCATCTCTTCATTATGCCAG-3'. *HOI*: 5'-GAGACGGCTTCAAGCTGGTGATG-3' and 5'-GTTGAGCAGGAACGCACTCTTGG-3'. *NQO1*: 5'-ATGGTCGGCAGAAGAGCACTGATCG-3' and 5'-TTTCTAGCTTTGATCTGGTTGTCACTTGGG-3'. Catalase: 5'-AAGGTTTGGCCTCACAAGG-3' and 5'-CCGCAATGTTCTCACACA-G-3'. Superoxide dismutase (*SOD*): 5'-ATGGCGACGAAGCCGTGTGCGTG-3' and 5'-TTGGCGCATCCCAATTA-CACCACAAG-3'. To ensure equal loading, total RNA was amplified with a set of  $\beta$ -actin primers: 5'-GCTCGTCGTCGACAACGGCTC-3' and 5'-CAAA-CATGATCTGGGTCATCTTCTC-3'. The temperature conditions were 50°C/1 h–94°C/5 min, followed by 25 cycles of 94°C/30 s–68°C/30 s–68°C/60 s and an extension of 68°C/2 min. RT-PCR products were visualized on ethidium bromide–stained 1.2% agarose gels.

**Cell proliferation assay.** The cell viability–proliferation assay was done using a CellTiter 96 Aqueous nonradioactive cell proliferation assay kit

(Promega Corporation). The assay is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] and an electron coupling reagent (phenazine methosulfate). MTS is bio-reduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm is then measured directly from 96-well assay plates.

**Statistical analysis.** Data are presented as the mean  $\pm$  SD. The Student's *t* test and one-way ANOVA were used to assess the significance of independent experiments.  $P < 0.05$  represents the statistical significance.

## Results

**Expression of NRP/B in breast cancers.** Breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, T-47D, and MDA-MB-231) were analyzed for *NRP/B* mRNA and protein expression by Northern and Western blot analyses, respectively. The human colon cancer cell line (HCT-116), which was reported to express NRP/B (31), served as a positive control. As shown in Fig. 1, *NRP/B* mRNA expression was detected in all cell lines. The level of NRP/B expression level in the HCT-116 cells was higher than that in the MCF-10A,



MCF-7, and T-47D cells. Interestingly, both the transcriptional and translational levels of NRP/B were abundant in MDA-MB-231 breast cancer cells. CSK was used as an internal loading control.

Immunohistochemistry showed that NRP/B was localized specifically in the nucleus of the cells (Fig. 2A), as previously shown in neuronal cells (22). Because MDA-MB-231 cells abundantly expressed NRP/B, we selected MDA-MB-231 breast cancer cell line as a model system to further analyze the biological function of NRP/B in breast cancer cells. To confirm the nuclear localization of NRP/B, cytoplasmic and nuclear extraction was done in MDA-MB-231 cells using NE-PER nuclear and cytoplasmic extraction reagents. Cellular extracts were subjected to Western blot analyses using VD2, anti-Lamin B1 (nuclear marker), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cytoplasmic marker) antibodies. NRP/B expression was strongly observed in the nuclear extract (Fig. 2B).

We then examined the localization of Nrf2 in breast cancer cells. Nrf2 was expressed in the cytoplasm, but more intensively in the nucleus of the MDA-MB-231, MCF-7, and T-47D cells (Fig. 2C). We also examined the expression of NRP/B and Nrf2 in human breast tissues by immunohistochemistry staining. NRP/B expression was localized in the nucleus and more intensively in the perinucleus (Fig. 2D). The expression pattern of Nrf2 was observed to be similar to that of NRP/B in breast cancer tissues (Fig. 2D).

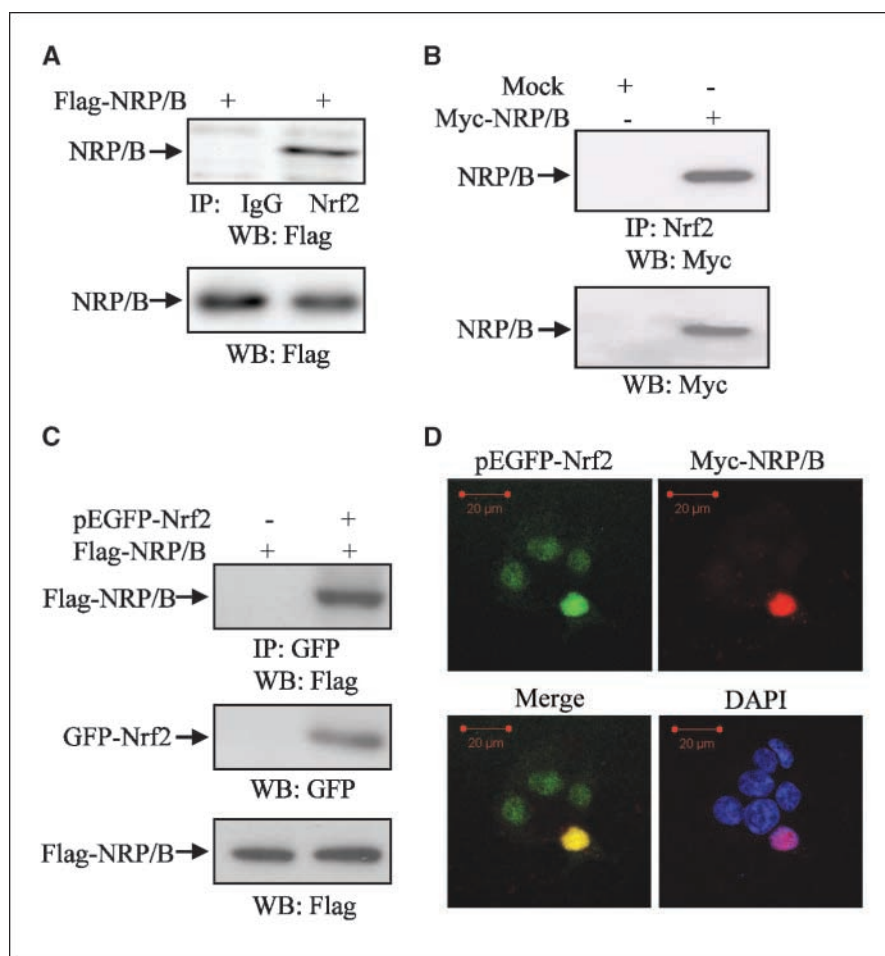
**In vitro and in vivo interaction of NRP/B with Nrf2.** 293T cells were transfected with Flag-NRP/B and immunoprecipitation was

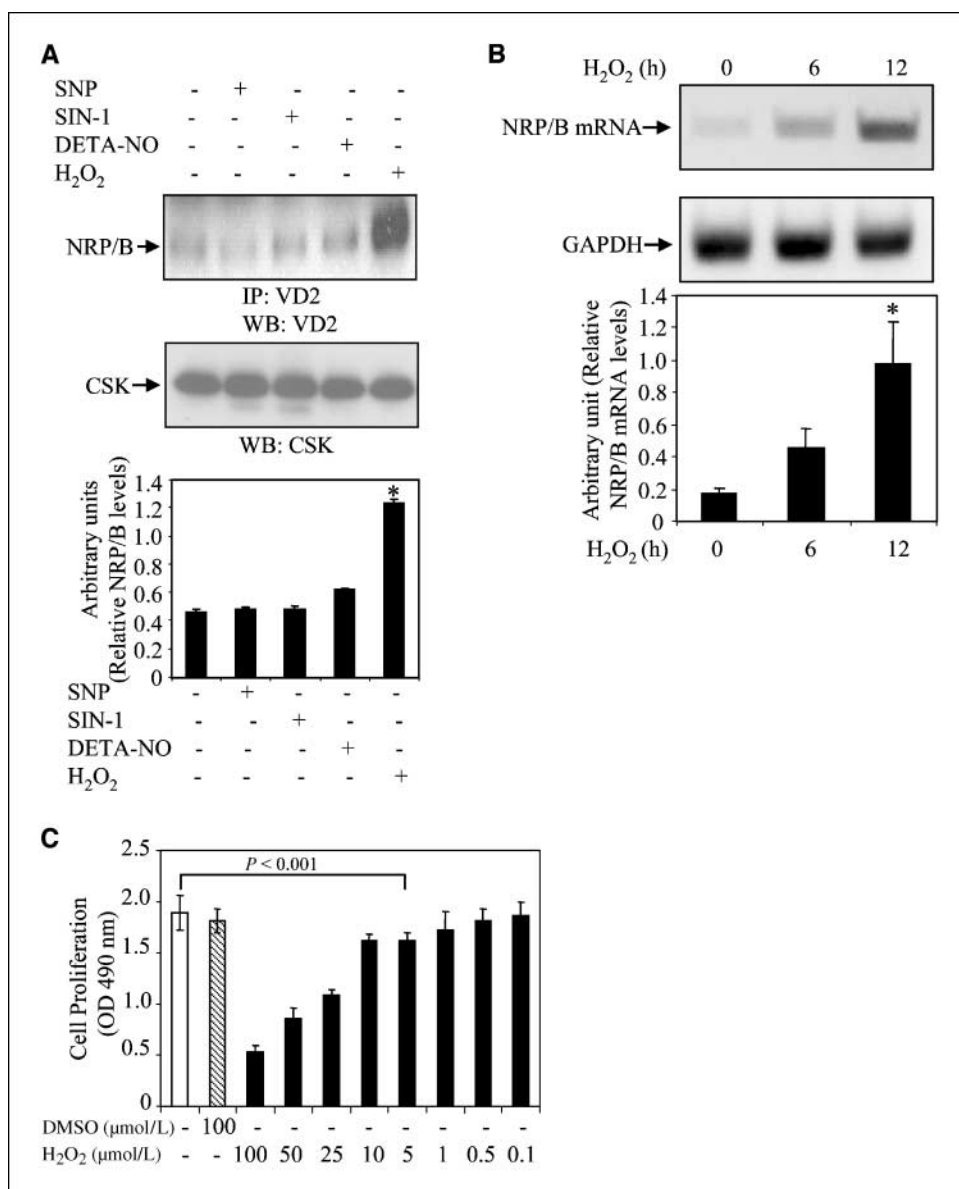
done using anti-Nrf2 antibody. The association of Nrf2 with NRP/B was detected by using anti-Flag antibody (Fig. 3A). A similar association of NRP/B with endogenous Nrf2 was observed when 293T cells were transfected with Myc-tagged NRP/B (designated as Myc-NRP/B) and then immunoprecipitated with anti-Nrf2 antibody and probed with anti-Myc antibody (Fig. 3B). We next did a pull-down assay to determine the association of both molecules. 293T cells were transfected with pEGFP-Nrf2, and the pull-down assay was done using recombinant GST-NRP/B protein as a bait. Nrf2 interacted with NRP/B as detected with anti-GFP antibody (Supplementary Fig. S1A). Similar experiments were carried out using anti-Nrf2 antibody (Supplementary Fig. S1B). MDA-MB-231 cells were also cotransfected with Flag-NRP/B and pEGFP-Nrf2. As shown in Fig. 3C, a complex consisting of Nrf2 and NRP/B was detected by the anti-Flag antibody, indicating that NRP/B interacted with Nrf2 in MDA-MB-231 cells.

**In vitro cellular sublocalization of NRP/B and Nrf2.** 293T cells were cotransfected with Myc-NRP/B and pEGFP-Nrf2 constructs, and then were stained with anti-Myc antibody and 4',6-diamidino-2-phenylindole (DAPI). As shown in Fig. 3D (merge), Myc-NRP/B and EGFP-Nrf2 were colocalized in the nucleus.

**Up-regulation of NRP/B expression in response to oxidative stress agents.** For these studies, the effects of NO donors (SNP, SIN-1, DETA-NO) and H<sub>2</sub>O<sub>2</sub> (9, 32) were tested in breast cancer cells. To optimize the concentration of SNP, SIN-1, DETA-NO, and H<sub>2</sub>O<sub>2</sub>, MDA-MB-231 cells were placed onto 96-well plates and treated with

**Figure 3.** Interaction and colocalization of NRP/B and Nrf2. 293T cells were transfected with DNA constructs, and protein extracts were prepared on the following day for the interaction analyses. **A**, 293T cells were transfected with the Flag-NRP/B construct. Cell extracts were immunoprecipitated with anti-Nrf2 or IgG antibodies and blotted with anti-Flag antibody. **B**, 293T cells were transfected with Myc-NRP/B or mock (pCMV-Myc). Cell extracts were immunoprecipitated with anti-Nrf2 antibody and blotted with anti-Myc antibody. **C**, MDA-MB-231 cells were cotransfected with the pEGFP-Nrf2 and Flag-NRP/B constructs. Cell extracts were immunoprecipitated with anti-GFP antibody and blotted with anti-Flag antibody. **D**, subcellular colocalization of NRP/B and Nrf2. 293T cells were cotransfected with the pEGFP-Nrf2 and Myc-NRP/B constructs. The cells were fixed with 4% paraformaldehyde, blocked with 5% skim milk, and incubated with anti-Myc antibody. Texas red-labeled secondary antibody was used to detect Myc-NRP/B expression. Merge and DAPI indicate nuclear colocalization of EGFP-Nrf2 and Myc-NRP/B. Bar, 20  $\mu$ m.





**Figure 4.** Up-regulation of NRP/B expression in response to H<sub>2</sub>O<sub>2</sub> in MDA-MB-231 cells. **A**, effect of H<sub>2</sub>O<sub>2</sub> on the growth of MDA-MB-231 cells. MDA-MB-231 cells were grown on 96-well plates and treated with H<sub>2</sub>O<sub>2</sub>, as indicated. A cell proliferation assay was done after 12 h of H<sub>2</sub>O<sub>2</sub> treatment using the cell proliferation kit, as indicated in Materials and Methods. **B**, top, MDA-MB-231 cells were treated with oxidative stress agents [SNP (2 mmol/L), SIN-1 (20 μmol/L), DETA-NO (10 μmol/L), and H<sub>2</sub>O<sub>2</sub> (10 μmol/L)] for 12 h, and protein extracts were prepared. Immunoprecipitation (IP) and Western blotting were done using anti-NRP/B (VD2) antibody. CSK served as an internal control for loading. **B**, bottom, relative NRP/B expression levels versus CSK levels in response to different oxidative stress agents (as derived from three individual experiments) were converted to arbitrary units. **C**, top, RT-PCR analysis of NRP/B mRNA expression in H<sub>2</sub>O<sub>2</sub>-treated MDA-MB-231 cells. The cells were treated with H<sub>2</sub>O<sub>2</sub> (10 μmol/L) for 0, 6, and 12 h. Total RNA was purified and subjected to PCR amplification using NRP/B-specific primers and GAPDH primers (internal control). **C**, bottom, relative NRP/B mRNA levels versus GAPDH levels upon H<sub>2</sub>O<sub>2</sub> treatment, as derived from three individual experiments, were converted into arbitrary units. Columns, mean; bars, SD. \*,  $P < 0.05$ .

various amounts of these chemicals for 24 h. Cell viability was then assessed by using the CellTiter Non-Radioactive Cell Proliferation Assay. Chemical concentrations that are considered as optimal should have no significant effect on cell proliferation and should be able to induce Nrf2-mediated downstream targets. As shown in Fig. 4A, treatment of cells with 25 to 100 μmol/L of H<sub>2</sub>O<sub>2</sub> induced a profound inhibitory effect on cell growth, whereas treatment with 5 to 10 μmol/L of H<sub>2</sub>O<sub>2</sub> had no significant effect on cell proliferation. In addition, 10 μmol/L of H<sub>2</sub>O<sub>2</sub> effectively induced Nrf2-mediated target genes. Thus, 10 μmol/L of H<sub>2</sub>O<sub>2</sub> was used as the optimal concentration for further analysis of NRP/B-mediated effects on oxidative stress responses. Optimal concentrations of SNP, SIN-1, and DETA-NO were 2 mmol/L, 20 μmol/L, and 10 μmol/L, respectively (data not shown). MDA-MB-231 cells were treated with SNP, SIN-1, DETA-NO, or H<sub>2</sub>O<sub>2</sub>. After 12 h of treatment, total cell lysates were prepared and immunoprecipitated with anti-NRP/B (VD2) antibody. H<sub>2</sub>O<sub>2</sub> treatment induced up to a 3-fold increase in NRP/B expression, whereas other oxidative agents had no effect on the

expression of NRP/B (Fig. 4B). We also examined the effect of H<sub>2</sub>O<sub>2</sub> on NRP/B transcript levels. MDA-MB-231 cells were treated with H<sub>2</sub>O<sub>2</sub> for a time course of 12 h. RNA was then purified and subjected to RT-PCR analysis. The results showed that NRP/B mRNA transcripts were up-regulated upon H<sub>2</sub>O<sub>2</sub> treatment over the 12 h time course (Fig. 4C). To further confirm that H<sub>2</sub>O<sub>2</sub> induced NRP/B expression, we used catalase to abolish the effect of H<sub>2</sub>O<sub>2</sub> on NRP/B. MDA-MB-231 cells were treated with 10 μmol/L of H<sub>2</sub>O<sub>2</sub> or with 10 μmol/L of H<sub>2</sub>O<sub>2</sub> plus catalase (10 μg/mL). As shown in Supplementary Fig. S2A and B, catalase seems to have a negative effect on H<sub>2</sub>O<sub>2</sub>, and subsequently H<sub>2</sub>O<sub>2</sub> failed to induce NRP/B expression.

**Increased association of NRP/B and Nrf2 upon H<sub>2</sub>O<sub>2</sub> treatment.** To determine the effect of H<sub>2</sub>O<sub>2</sub> on the association of Nrf2 and NRP/B, we did a short time course of H<sub>2</sub>O<sub>2</sub> induction in 293T cells transfected with Myc-NRP/B. An increased association of Myc-NRP/B and endogenous Nrf2 was observed 1 h after treatment of 10 μmol/L of H<sub>2</sub>O<sub>2</sub> compared with the untreated control, and this increased association between NRP/B and Nrf2

declined 6 h after treatment (see Supplementary Fig. S3A). To investigate the effect of  $H_2O_2$  treatment on the endogenous association of NRP/B with Nrf2 in MDA-MB-231 cells, cells were exposed to  $H_2O_2$  and the association of these proteins was analyzed. As shown in Fig. 5A, an increased interaction between NRP/B and Nrf2 was observed in the MDA-MB-231 cells.

**Colocalization of NRP/B and Nrf2 upon  $H_2O_2$  treatment.** MDA-MB-231 cells were treated with  $H_2O_2$  for 12 h, fixed, and immunostained with anti-Nrf2 and anti-NRP/B (VD2) antibodies. The Nrf2 and NRP/B expression levels were increased and the colocalization of Nrf2 and NRP/B became more intense in the nucleus of these cells after  $H_2O_2$  treatment (Fig. 5B).

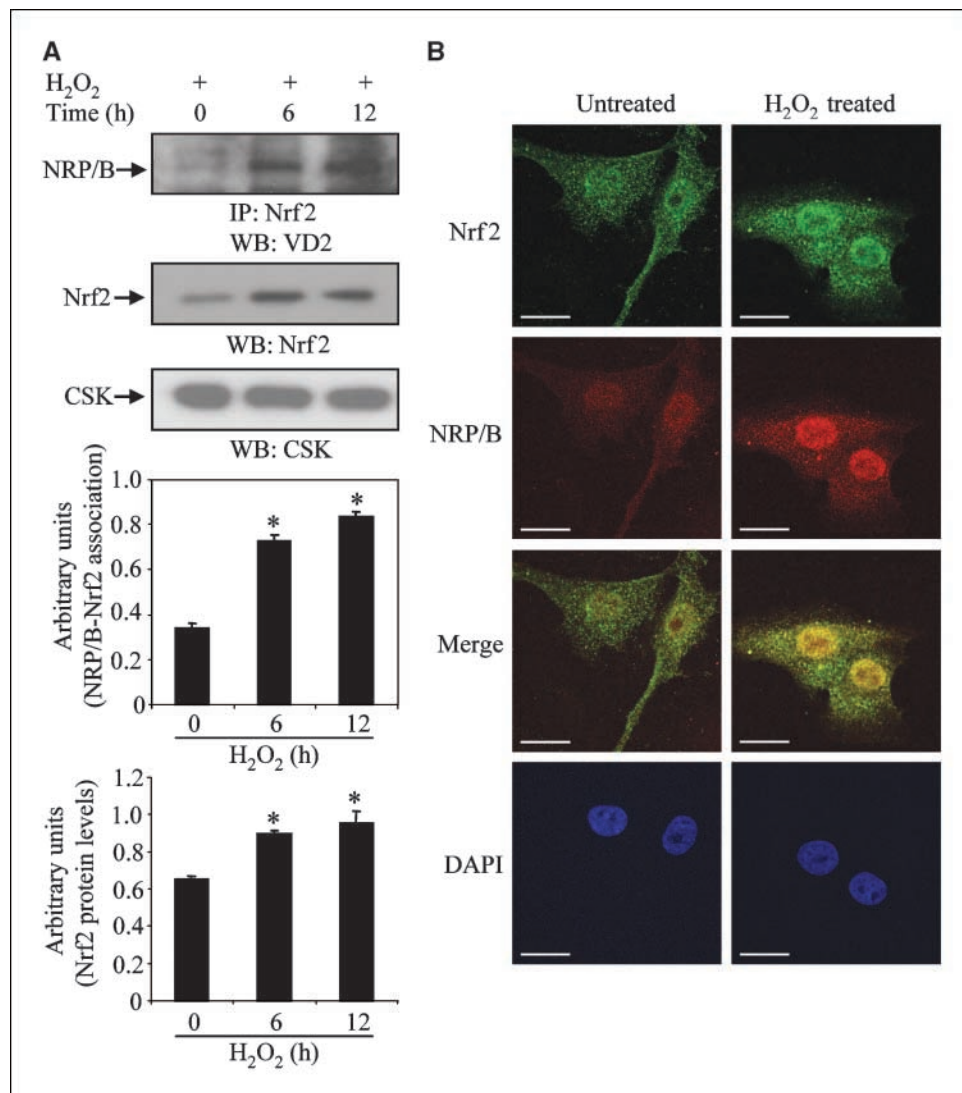
**$H_2O_2$  induces the expression of Nrf2-mediated target genes.** MDA-MB-231 cells were treated with  $H_2O_2$  and total mRNA was prepared. The expressions of *TRX*, heme oxygenase (*HO-1*), *NQO1*, catalase, and *SOD* were determined by semiquantitative RT-PCR. A significant increase in *TRX* and *NQO1* expression levels was observed after  $H_2O_2$  stimulation (Fig. 6A). However, there were no significant effects on *HO-1*, catalase, and *SOD* expression upon  $H_2O_2$  treatment compared with the untreated control. Thus, both *TRX* and *NQO1* are downstream targets of the NRP/B-Nrf2

interaction, which may confer protection against  $H_2O_2$ -induced toxicity in MDA-MB-231 cells.

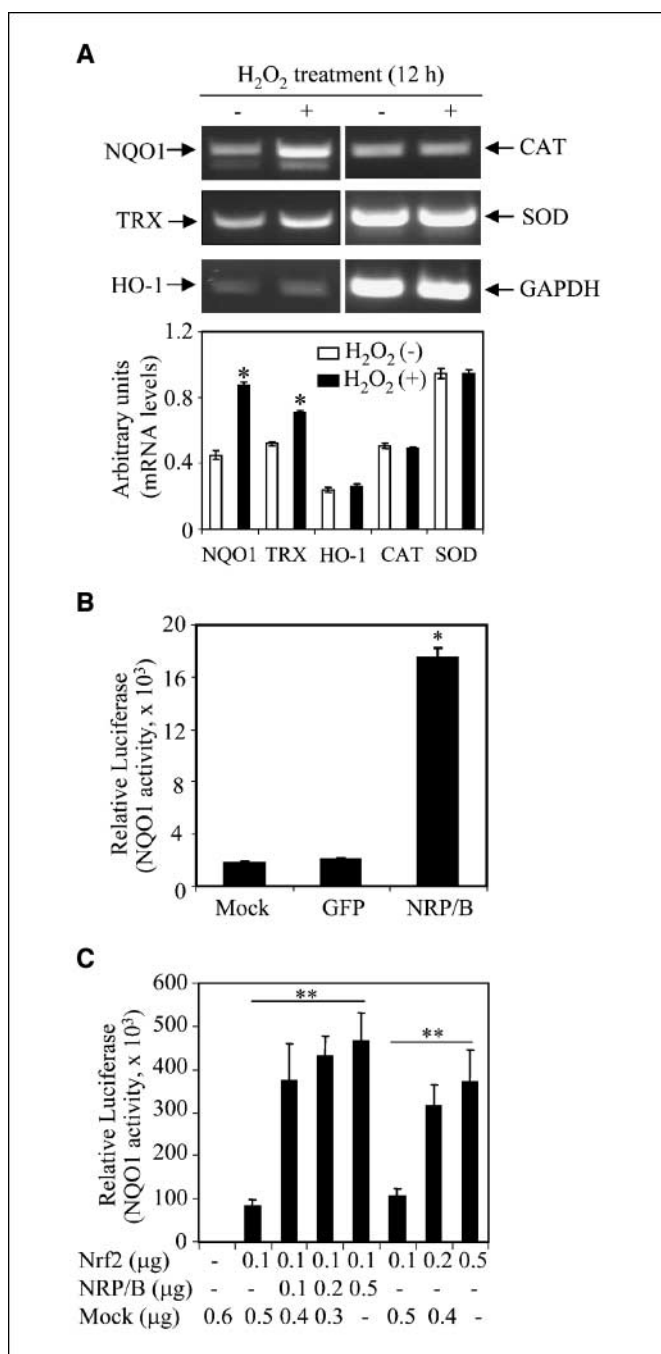
**NRP/B enhances the activity of the NQO1 promoter.** The ARE-*NQO1* promoter sequence (−1,016/*nqo5*) constructed with the pGL3-basic luciferase reporter vector was used to evaluate the effect of NRP/B on NQO1 activity. 293T cells were cotransfected with the *NQO1* promoter and mock (pCMV-Myc), pEGFP-C2, or Myc-NRP/B. Following 24 h of transfection, total cell lysates were prepared and luciferase activity was measured. Relative luciferase activity was ~9-fold higher in the 293T cells transfected with Myc-NRP/B compared with cells receiving the mock or GFP transfections (Fig. 6B). An ~2-fold increase in relative luciferase activity was observed in COS7 cells under similar transfection conditions (see Supplementary Fig. S3B).

Furthermore, we examined the effect of Nrf2 on *NQO1* promoter activity. 293T cells were transfected with various concentrations of Nrf2. The Nrf2 treatment increased NQO1 activity in a dose-dependent manner (Fig. 6C). To examine the effect of NRP/B on Nrf2 regulation of NQO1 activity, 293T cells were transfected with 0.125  $\mu$ g of *Nrf2* (as a baseline) and with various doses of *NRP/B*. A significant increase in relative luciferase activity (up to 5-fold) was

**Figure 5.** Effect of  $H_2O_2$  on the association and localization of NRP/B and Nrf2. **A, top,** MDA-MB-231 cells were treated with  $H_2O_2$  (10  $\mu$ mol/L) over a time course, as indicated. Total cell lysates were subjected to immunoprecipitation with anti-Nrf2 antibody followed by Western blotting using anti-NRP/B (VD2) antibody. **Middle and bottom,** expression levels of Nrf2 and CSK as analyzed by Western blotting with anti-Nrf2 and anti-CSK antibodies, respectively. Relative levels of the NRP/B and Nrf2 association (**A, middle**), and Nrf2 expression (**A, bottom**), as derived from three independent experiments, were converted into arbitrary units. **Columns,** mean; **bars,** SD. \*,  $P < 0.05$ . **B,** MDA-MB-231 cells were grown on coated slide glasses and then untreated or treated with  $H_2O_2$  (10  $\mu$ mol/L), as indicated. The slide glasses were fixed with 4% paraformaldehyde and treated with 0.5% Triton X-100. The slides were incubated with anti-Nrf2 and anti-NRP/B (VD2) antibodies. Subsequently, secondary antibodies conjugated with FITC and Tris were added. **Green and red,** expression of Nrf2 and NRP/B, respectively. **Merge,** colocalization of NRP/B and Nrf2. DAPI staining indicates the nucleus. The images were taken using a confocal microscope (Zeiss LSM 510 Meta). **Bar,** 20  $\mu$ m.







**Figure 6.** Effect of NRP/B on Nrf2-mediated gene expression. **A**, top, Nrf2-mediated expression of genes in MDA-MB-231 cells upon H<sub>2</sub>O<sub>2</sub> treatment. Cells were cultured and treated with H<sub>2</sub>O<sub>2</sub> (10 μmol/L) for 12 h. Total RNA was purified and subjected to semiquantitative RT-PCR analysis. *TRX*, *HO-1*, *NQO1*, catalase, and *SOD* expressions were then measured. *CAT*, catalase. **A**, bottom, the relative mRNA expression levels of Nrf2-target genes versus GAPDH levels upon H<sub>2</sub>O<sub>2</sub> treatment, based on three individual experiments, are shown in arbitrary units. \*, *P* < 0.05. **B**, effect of NRP/B on *NQO1* promoter activity. 293T cells were cotransfected with the *NQO1* promoter and Myc-NRP/B, pEGFP-C2, or pCMV-Myc (Mock). After 24 h, total lysates were prepared, *NQO1* promoter activity (luciferase activity) was measured, and then the relative luciferase activity (ratio of luciferase and β-gal activities) was expressed. **C**, effect of NRP/B on *NQO1* activity via the Nrf2 pathway. 293T cells were transfected with Nrf2 (0.1 μg) and various doses of NRP/B (0–0.5 μg). In a parallel experiment, 293T cells were transfected only with Nrf2 in a dose-dependent manner (0–0.5 μg). After 24 h, total lysates were prepared, *NQO1* promoter activity (luciferase activity) was measured, and then the relative luciferase activity (ratio of luciferase and β-galactosidase activities) was expressed. Columns, mean; bars, SD. \*, *P* < 0.05, \*\*, *P* < 0.01.

found in cells cotransfected with *Nrf2* and *NRP/B* (Fig. 6C). Taken together, these data (Fig. 6C), along with the results presented in Figs. 4 and 5, indicate that NRP/B enhances NQO1 activity via the Nrf2 pathway.

## Discussion

The current study reports the involvement of the nuclear matrix protein NRP/B in Nrf2-mediated oxidative stress responses in breast cancer cells. This work shows that the association of NRP/B with Nrf2 is important in the activation of Nrf2-mediated *NQO1* transcription, thereby conferring protection against oxidative stress-induced damage in breast cancer cells.

Protective or oxidative stress responses to extra and/or intracellular environmental stresses are important for the physiologic activity of cells. One of the oxidative stress response pathways is mediated by Nrf2 (33). Although there is growing evidence that the Nrf2 pathway is involved in cellular defenses in the brain, retinal epithelia, liver, lungs, gastrointestinal tract, kidney, spleen, and erythrocytes (13), the involvement of Nrf2-mediated defenses in breast cancer is largely unknown. Here, we show the association of NRP/B with Nrf2, a requirement for the activation of Nrf2-downstream target genes in breast cancer, indicating an important role of NRP/B in oxidative stress responses via Nrf2.

NRP/B plays important roles in various biological processes such as central nervous system development (21, 22) and neuronal outgrowth (21, 23, 34) as well as contributes to brain tumors (23), hairy cell leukemia (25), and colorectal carcinogenesis (35). In our study, NRP/B expression was observed in breast cancer cell lines and tissues (Figs. 1 and 2). Although NRP/B was also detected in breast epithelial cells such as MCF-10A cells, its expression level was very low (Fig. 1A and B), suggesting that NRP/B may have important biological function(s) in breast cancer cells, particularly in MDA-MB-231 cells, which are highly aggressive and form metastasis in mice.

In response to oxidative stress-induced damage, Nrf2 is released by its transcriptional repressor Keap1, whereupon it translocates to the nucleus and activates downstream target genes. Our studies showed that to activate Nrf2-mediated target genes, Nrf2 is required to be associated with NRP/B, and subsequently to have its action magnified, thereby boosting phase II detoxifying enzymes and antioxidant proteins such as NQO1. Although Keap1 and NRP/B share the common feature of binding to Nrf2, their differing functions in the Nrf2 pathway may be due to varying subcellular localization, where Keap1 is localized in the cytosol and NRP/B is localized in the nucleus. Thus, NRP/B may play a crucial role in the nuclear compartment in response to oxidative stress-induced damage.

Oxidative stress-inducing factors disrupt the Keap1-Nrf2 complex and Nrf2 translocation to the nucleus (7, 36). Hence, we tested whether the nitric oxide (NO) donors (SNP, SIN-1, DETA-NO; ref. 15) and the oxidizing agent H<sub>2</sub>O<sub>2</sub> (32), which induce cellular damage, exert any effects on the NRP/B-Nrf2 interaction in breast cancer cells. H<sub>2</sub>O<sub>2</sub> was the only agent that up-regulated NRP/B expression and induced the association of NRP/B with Nrf2 (Figs. 4 and 5), suggesting that this NRP/B-Nrf2 complex may be involved in regulating ARE-driven genes to protect cells from H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity.

*NQO1* transcription is activated in response to xenobiotics and antioxidants (9), and protects breast cancer cell lines from H<sub>2</sub>O<sub>2</sub>-induced damage (32). Therefore, activation of *NQO1* transcription may protect MDA-MB-231 cells against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity (Fig. 6A). We observed that Nrf2 induced *NQO1* promoter activity

and that this promoter activity was enhanced up to 5-fold when Nrf2 was cotransfected with NRP/B in a dose-dependent manner (Fig. 6C). As shown in Fig. 4A, NRP/B expression level was increased in response to H<sub>2</sub>O<sub>2</sub> treatment. Hence, NRP/B seems to be essential for Nrf2-mediated *NQO1* expression in response to H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity.

NO is a reactive free radical participating in several types of redox reactions, which mediate its biological effects or limit its activity in specific chemical reaction(s). In combination with superoxide, NO forms a reactive peroxynitrite anion that is able to nitrate and oxidize protein residues, DNA, and lipids, thereby affecting cellular homeostasis (37). Although NO affects cellular homeostasis (37), NO donors SNP, DETA-NO, and SIN-1 (referred to as peroxynitrite generators; ref. 38) had no effect on NRP/B expression in MDA-MB-231 cells (Fig. 4). Moreover, treatment of MDA-MB-231 cells with peroxynitrite exerted no effect on NRP/B expression and NRP/B-Nrf2 association (data not shown). Thus, NO donors may be irrelevant to the activation of oxidative stress responses via the NRP/B-Nrf2 pathway in MDA-MB-231 cells.

H<sub>2</sub>O<sub>2</sub> is a biologically important oxidant, generating highly reactive hydroxyl free radicals through its interaction with redox-active transitional metals. Cellular concentrations of H<sub>2</sub>O<sub>2</sub> are one of the major factors contributing to pathologic consequences. At low concentrations (<10 μmol/L), H<sub>2</sub>O<sub>2</sub> was shown to up-regulate certain proteins and to stimulate the proliferation of mammalian cells (39). However, elevated levels of H<sub>2</sub>O<sub>2</sub> induced oxidative stress or injury to the cells (39) and exerted increasingly inhibitory effects

on cell proliferation in a concentration-dependent manner (Fig. 4). H<sub>2</sub>O<sub>2</sub> also induced NRP/B expression and up-regulated the NRP/B-Nrf2 interaction. Therefore, NRP/B up-regulation is involved in oxidative stress responses rather than in cell growth. Furthermore, whereas H<sub>2</sub>O<sub>2</sub> played a crucial role in mounting oxidative stress responses through the NRP/B-Nrf2 pathway in breast cancer cells, NO donors seemed to have no effect on this pathway. The distinction between NO donors and H<sub>2</sub>O<sub>2</sub> may be due to the nature of their biochemical reactions and cell type specificity, and may correlate with the ability of each oxidative insult to activate the NRP/B-Nrf2 system in breast cancer cells.

Collectively, the NRP/B-Nrf2 association is essential for activation of Nrf2-mediated *NQO1*. This antioxidant enzyme was reported to be used in cancer prevention (40–42) and in enzyme-directed tumor targeting (40, 43). The inducer(s)/activator(s) of *NQO1* may represent efficient protective molecule(s) for therapeutic intervention in human breast cancer. Taken together, NRP/B plays an important role in Nrf2-mediated oxidative stress responses, and may serve as a potential anticancer drug candidate for breast cancer.

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## The Nuclear Matrix Protein, NRP/B, Enhances Nrf2-Mediated Oxidative Stress Responses in Breast Cancer Cells

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