Supporting Information

De Luca et al. 10.1073/pnas.1010171107

SI Materials and Methods

MDA-MB231 Clone Selection. MDA-MB231 transfected cells were puromycin selected (1 μg/mL) and independently tested for down-regulation of MsrA expression. The most effective vector (TI363690) was then transfected under puromycin selection to generate a stable cell line (pRS-shMsrA), and was compared with MDA-MB231 cells stably transfected (in parallel) with an empty shRNA vector (pRS) or with GFP-directed shRNA (pRS-shGFP). After 3 wk, single independent clones were randomly isolated, and each of the individual clones (sh1-MsrA, sh2-MsrA, sh3-MsrA) was plated separately. After clonal expansion, cells from each independent clone were tested for MsrA expression by RT-PCR and Western blotting. Experiments were performed with several independent clones to avoid clonal effects.

Mock and silenced MDA-MB231 cells were plated into six-well plates and cultured until 80% confluence. The cells were harvested, and total RNA was extracted, and reverse transcription was performed as described below (real-time PCR). To evaluate the effective silencing of MsrA, the F1MsrA, R1MsrA, GAPDHf, and GAPDHr primers (Table S2) were used as previously described (1). The abundance of PCR products was evaluated by densitometric scanning of the ethidium bromide-stained agarose gels using the Chemi Doc System (Bio-Rad). Cells from each independent clone were tested for MsrA expression at the protein level using an anti-MsrA antiserum prepared as previously described (1). MsrB1, MsrB2, MsrB3a, and MsrB3b mRNA levels in mock and silenced MDA-MB231cells were evaluated by RT-PCR (Table S2).

PTEN mRNA levels were analyzed using fPTEN and rPTEN primers (Table S2).

Determination of Cellular ROS. Cells were plated in six-well plates (5×10^5/well) in full medium without phenol red. After 24 h, the culture medium was replaced with new medium containing the ROS-sensitive fluorescent dye DCFDA (10 μM) and incubated 30 min in the dark at 37 °C. Cells were then detached by trypsinization, collected by centrifugation, and washed twice in PBS, centrifuged for 5 min at 1,000 rpm, and finally resuspended on 200 μL cold PBS. The fluorescence intensity that reflected the level of intracellular ROS was measured using a Becton-Dickinson FACS-Calibur flow cytometer.

ECM Degradation Assay. Fluorophore-conjugated gelatin was prepared according to published procedures (2, 3), with thin layers of rhodamine B isothiocyanate (Sigma-Aldrich) conjugated porcine gelatin (Sigma-Aldrich) placed on coverslips, cross-linked with 0.5% glutaraldehyde for 15 min on ice, washed three times with PBS, and incubated for 3 min at room temperature with 5 mg/mL $NaBH_4$. Finally, after three washes with PBS and a 5-min incubation in 70% ethanol, the coverslips were maintained with complete medium for 1 h at 37 °C before cell plating. The cells were plated directly onto the gelatin-coated coverslips; after an overnight incubation, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized in PBS containing 0.02% saponin, 0.2% BSA, and 50 mM NH_4Cl, and incubated with fluorophore-conjugated phalloidin (Molecular Probes) for 1 h. Finally, the coverslips were mounted in Mowiol (Calbiochem). Experiments were assessed using an LSM510 laser scanning confocal microscope (Carl Zeiss). The areas of degradation were quantified considering at least 100 cells per condition using LSM510-3.2 software (Carl Zeiss). The total degradation area was then normalized for the number of cells.

Western Blotting and Antibodies. Protein extracts were obtained from 80% confluent cells, which were washed in cold PBS and lysed in lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, and 0.5% Nonidet P-40) containing Halt Protease Inhibitor Mixtures (Pierce), 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM sodium pyrophosphate. Protein concentrations were determined using the BCA assay (Pierce), with albumin as standard. Crude extracts were analyzed by SDS/PAGE on 12.5% (wt/vol) polyacrylamide gels, and the proteins were then transferred to nitrocellulose membranes (Bio-Rad). For detection of immunoreactive bands, Western blotting luminol reagent (sc-2048; Santa Cruz) was used. Western blots were quantified using National Institutes of Health ImageJ software integrated density analysis.

Goat polyclonal anti-VEGF (sc-1836), rabbit polyclonal anti-SOD2 (sc-30080), rabbit polyclonal anti-SOD1 (sc-11407), and rabbit polyclonal anti-GPx (sc-30147) antibodies were obtained from Santa Cruz. A rabbit polyclonal anti-catalase antibody (219010) was purchased from Calbiochem, a rabbit polyclonal anti-PTEN antibody from Cell Signaling Technology (9552), a mouse monoclonal anti–β-actin antibody from Sigma (A4700), and HRP-conjugated anti-rabbit (401393), antimouse (401253), or antigoat (401504) secondary antibodies from Calbiochem.

Cell Proliferation Assay and Cell Growth. Cells were seeded in 96-well plates at 1×10^3 cells per well in 100 μL culture medium and grown at 37 °C. At the indicated incubation times (4, 24, 48, and 72 h), 20 μL CellTiter 96 AQueous One Solution Reagent (Promega) was added to each well and the plates were incubated for 4 h at 37 °C to develop the formazan product. Subsequently, absorbance at 490 nm was measured using a 96-well plate reader. Also, 2×10^5 cells were seeded in 35-mm plates in complete medium at 37 °C, and incubated for 24, 48, 72, and 96 h; these cells were trypsinized and counted.

3D Cell Culture. Cells were detached from culture dishes, and 2×10^4 cells/100 μL DMEM containing 10% FCS were suspended in 250 μL Matrigel (BD Biosciences) solution at 4 °C, and then quickly added to a precoated Matrigel layer in 24-well plates. The top Matrigel cell layers were gelled at 37 °C for 60 min, and then covered with culture medium containing 10% FCS. The cells were then cultured at 37 °C. After 1 wk, the medium was discarded and 400 μL Dispase (Becton Dickinson) was added to each well, and the plates were incubated for 2 h at 37 °C. The digested material was centrifuged at $4,000 \times g$ for 10 min, and the pellet containing the cells was rinsed in prewarmed PBS and treated with trypsin for 5 min at 37 °C to recover single-cell suspensions. The cell suspensions were counted in triplicate.

VEGF Real-Time PCR. Total cellular RNA was isolated from stable transfectants using the SV Total RNA Isolation System (Promega), according to the manufacturer's instructions. Reverse transcription reactions were performed using 2 μg total RNA, in a final volume of 40 μL, using Moloney murine leukemia virus reverse transcriptase (Sigma) and poly(dT) primers, as recommended by the manufacturer. The product (50 ng) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT sequence detection system (Applied Biosystems).

A human-specific probe set was obtained from ABI for vascular VEGF A (HS00173626-m1). Comparisons were made using the $\Delta\Delta C_t$ method, with a probe set directed against human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, HS99999905-m1).

Xenograft Nude Mice Analysis. Total RNA was extracted from tumors and subjected to reverse transcription as reported above. PCR amplification was performed using F1MsrA, R1MsrA, GAPDHf, and GAPDHr primers (Table S2).

1. De Luca A, Sacchetta P, Di Ilio C, Favaloro B (2006) Identification and analysis of the promoter region of the human methionine sulphoxide reductase A gene. *Biochem J* 393:321–329.
2. Baldassarre M, et al. (2003) Dynamin participates in focal extracellular matrix degradation by invasive cells. *Mol Biol Cell* 14:1074–1084.
3. Bowden ET, Mueller S, Coopman PJ (2001) In vitro invasion assays: Phagocytosis of the extracellular matrix. *Current Protocols in Cytometry* (Wiley, New York), pp 9.13.1–9.13.8.

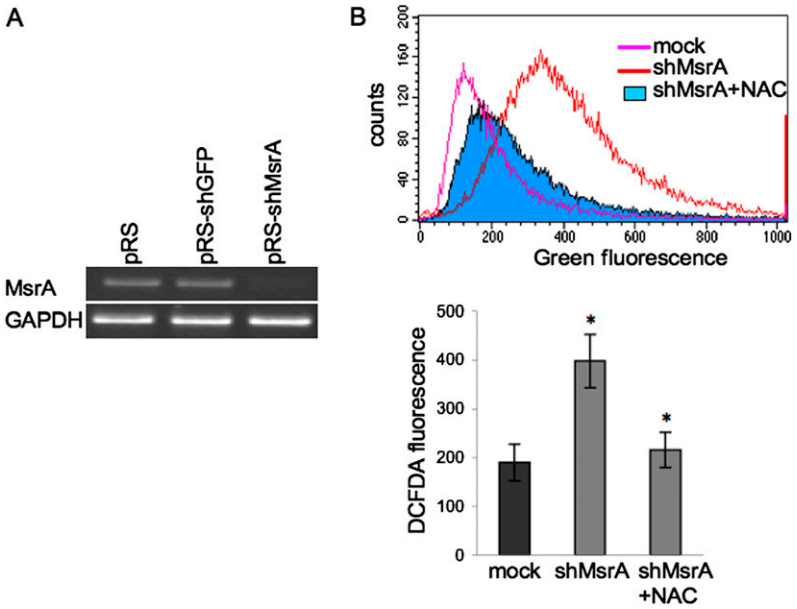

Fig. S1. (*A*) mRNA levels for MsrA analyzed by RT-PCR and described in *SI Materials and Methods*. (*B*) MDA-MB 231 mock and shMsrA cells were stained with 10 μM DCFDA and analyzed by flow cytometry, to determine the H_2O_2 production. Reduction of MsrA expression by shRNA led to increased ROS levels, compared with mock cells. The general ROS-quenching agent NAC (10 mM) reduced ROS levels induced by silencing of MsrA. (*Upper*) Representative experiment. (*Lower*) Quantification as means ± SD of three independent experiments. *$P < 0.01$ (Student *t* test).

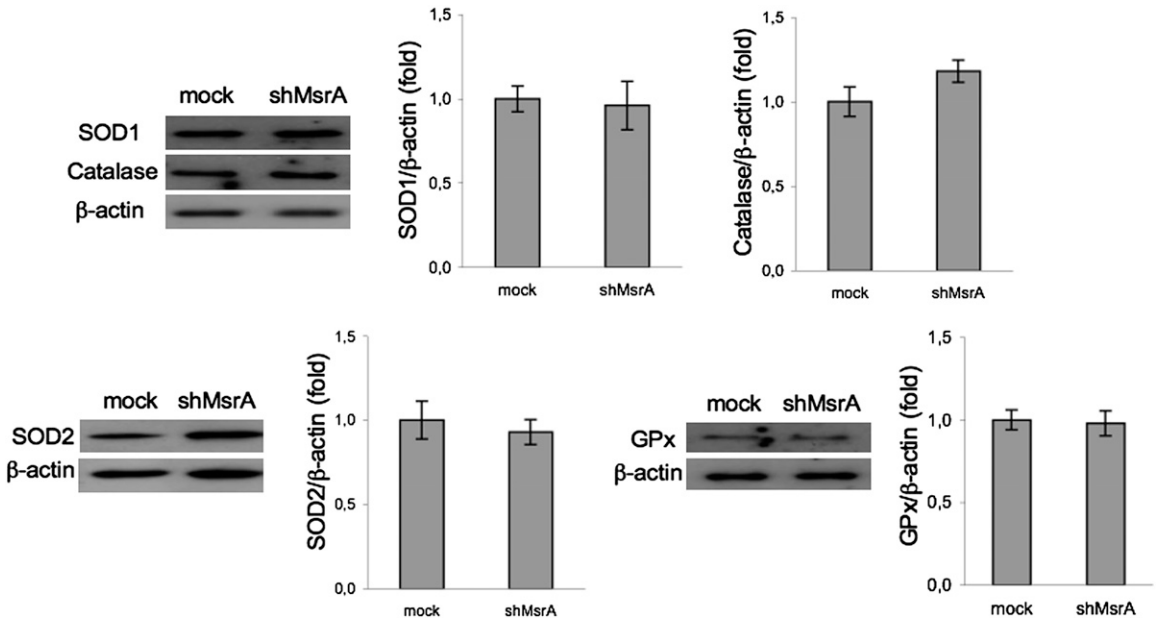

Fig. S2. Protein levels of the antioxidant enzymes analyzed by Western blotting: catalase, GPx, SOD1 and SOD2, as indicated (left). Equal amounts of protein (100 μg) were extracted from mock and shMsrA MDA-MB231 cells, using specific polyclonal antibodies. β-Actin was used to normalize protein loading. (Right) Quantification by densitometric analysis as means ± SD ($n = 3$).

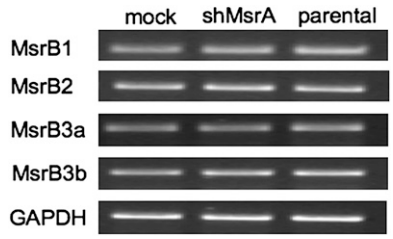

Fig. S3. mRNA levels for MsrB1, MsrB2, MsrB3a, and MsrB3b, as analyzed by RT-PCR (*SI Materials and Methods*).

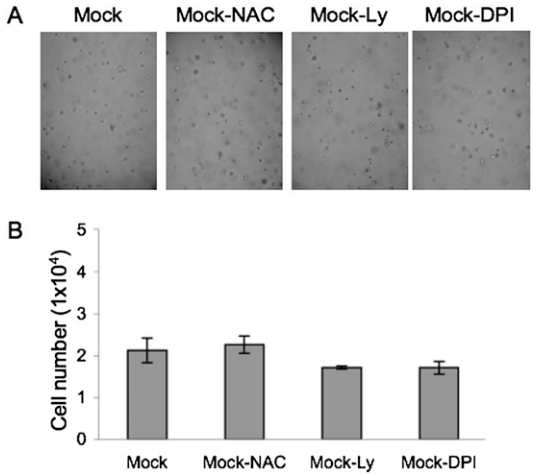

Fig. S4. (*A*) Representative phase-contrast microscopy images of mock cells in Matrigel seeded at 2 $\times 10^4$ cells per well in 24-well plates. Cells were grown for 1 wk in the presence of vehicle, 5 mM NAC, 10 μM LY294002, and 1 μM DPI. (*B*) Total cell numbers determined after Dispase digestion. Data are mean ± SD of three independent experiments.

Table S1. Specific MsrA-directed 29-mer oligos (Origene)

OriGene catalog number	Sequence
TI363689	CCTGTAGCGGCCAAACATCATGTCAATGG
TI363690	GAAAGGAGTGTATTCAACTCAAGTTGGTT
TI363691	GTCCGAGTGGTGTACCAGCCAGAACACAT
TI363692	ACTATGCGGAAGACTACCACCAGCAGTAC

Table S2. Sequences of all primers used in this study

Primer name	Sequence
F1MsrA	5′-GACCACCCTTCGGCTGG-3′
R1MsrA	5′-CCACCATGTGGGGAGCAA-3′
fMsrB	5′-ATGTCGTTCTGCAGCTTCTTC-3′
rMsrB	5′-CACACTTGCCACAGGACAC-3′
fMsrB2	5′-GCTCCTCTGGTTGCTCC-3′
rMsrB2	5′-AAAGCCACACTGTTGATGC-3′
1fMsrB3	5′-ATGAGCCCGCGGCGGAC-3′
2fMsrB3	5′-ATGTCTGCATTCAACCTGCTGCAT-3′
rMsrB3	5′-GCGGTGCCACTGCTATCC-3′
GAPDHf	5′-CCACCCATGGCAAATTCCATGGCA-3′
GAPDHr	5′-TCTAGACGGCAGGTCAGGTCCACC-3′
fPTEN	5′-ATGACAGCCATCATCAAAG-3′
rPTEN	5′-GTCCTTACTTCCCCATAG-3′