CANCER BIOLOGY & THERAPY
2024, VOL. 25, NO. 1, 2416221
https://doi.org/10.1080/15384047.2024.2416221

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

NRF3 suppresses the malignant progression of TNBC by promoting M1 polarization of macrophages via ROS/HMGB1 axis

Ping Xing[a,b], Zhenzhen Chen[c], Wenbo Zhu[a,b], Bangyi Lin[d], and Mingming Quan[d]

[a]Department of Surgical Oncology, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Taizhou, Zhejiang, China; [b]Department of Surgical Oncology, Enze Hospital, Taizhou Enze Medical Center, Taizhou, Zhejiang, China; [c]Department of Ultrasound, Taizhou Central Hospital (Taizhou University Hospital), Taizhou, Zhejiang, China; [d]Department of Surgical Oncology, Taizhou Central Hospital (Taizhou University Hospital), Taizhou, Zhejiang, China

ABSTRACT

Background: Triple-negative breast cancer (TNBC) is a highly aggressive form of breast cancer. Due to its lack of targeted therapy options, TNBC remains a significant clinical challenge. In this study, we investigated the role of nuclear respiratory factor 3 (NRF3) and high-mobility group box 1 (HMGB1) in the progression of TNBC.

Methods: The study analyzed NRF3's clinical expression, differentially expressed genes (DEGs), and immune infiltration in TNBC using the TCGA database and bioinformatics tools. Cellular functions of MDA-MB-468 and Hs578t cells were evaluated through MTT, colony formation, transwell, flow cytometry, and western blotting. The regulatory function of NRF3 in TNBC cell lines was assessed using Immunofluorescence, Immunohistochemistry, qRT-PCR, CHIP, luciferase assay, and ELISA. Moreover, a xenograft model was established to investigate the role of NRF3 in TNBC in vivo.

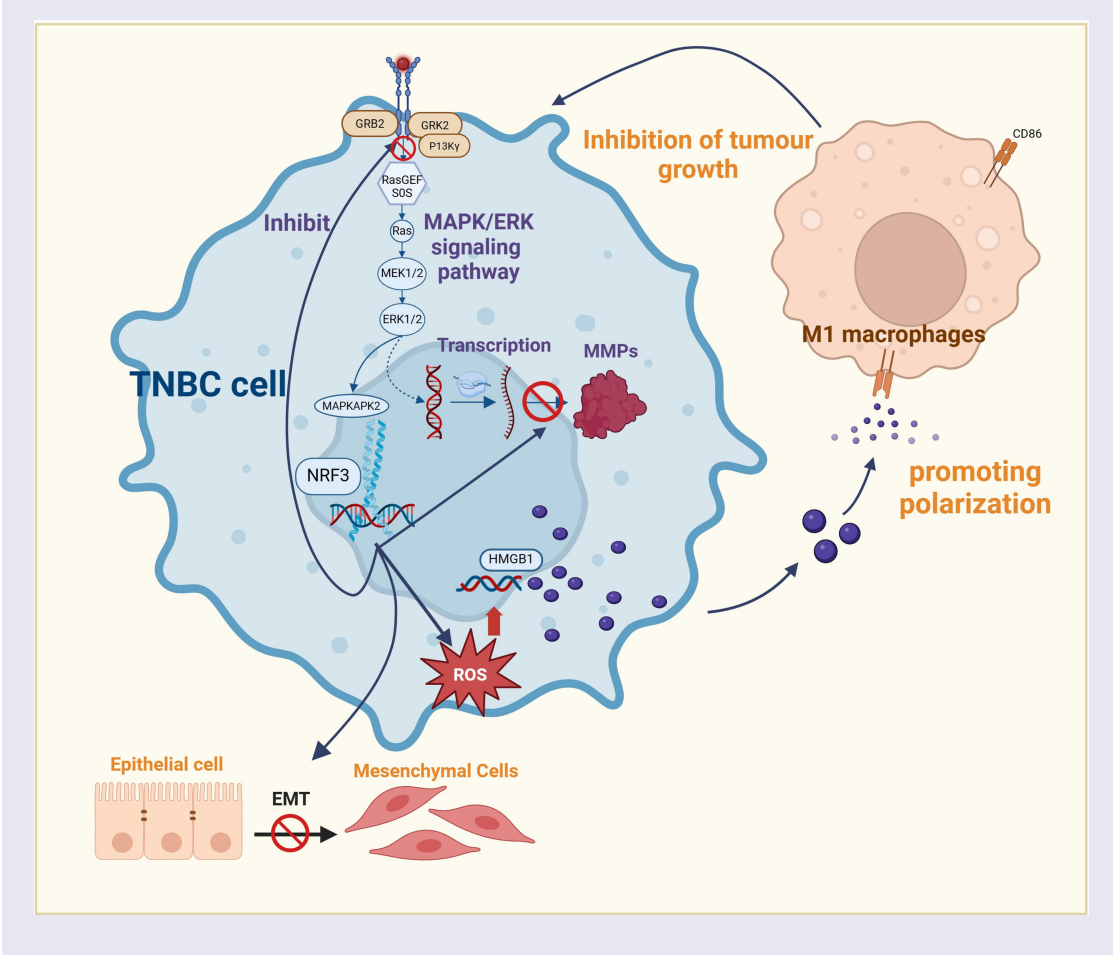
Results: Low expression of NRF3 in TNBC tumors was associated with unfavorable prognosis and transcripts from tumors with higher NRF3 levels were enriched in oxidative stress and immune-related pathways. The subsequent gain- and loss-functional experiments indicated that NRF3 overexpression significantly suppressed malignant phenotypes, MAPK/ERK signaling pathways, and epithelial-mesenchymal transition (EMT), whereas it promoted reactive oxygen species (ROS) levels in TNBC. Further mechanistic exploration showed that NRF3 inhibited TNBC cell function by regulating oxidative stress-related genes to inhibit the MAPK/ERK signaling pathway by promoting the release of HMGB1 via ROS, thereby promoting M1 macrophage polarization.

Conclusion: NRF3 promotes M1 macrophage polarization through the ROS/HMGB1 axis, thereby inhibiting the malignant progression of TNBC. It is expected to become a therapeutic biomarker for TNBC.

ARTICLE HISTORY
Received 8 March 2024
Revised 23 September 2024
Accepted 8 October 2024

KEYWORDS
Triple-negative breast cancer; NRF3; ROS; HMGB1; macrophages

CONTACT Mingming Quan aoxiangquan@163.com Taizhou Central Hospital (Taizhou University Hospital), Donghai Avenue 999, Taizhou, Zhejiang 318000
Supplemental data for this article can be accessed online at https://doi.org/10.1080/15384047.2024.2416221



1. Introduction

Breast cancer, a disease of high incidence and mortality rate, is the second most prevalent cancer among women globally, with over 2 million new cases and nearly 600,000 associated mortalities annually.[1,2] Triple-negative breast cancer, which represents 15–20% of all incident BC,[3] is a unique BC subtype due to its deficiency in targeted treatments, characterized by the lack of expression of three crucial markers, estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2).[4] TNBC is renowned for its poor prognosis, earning it the title of the "King of BC".[5] Furthermore, the tumor microenvironment (TME) plays a pivotal role in the progression and therapeutic response of TNBC. The TME comprises a diverse ensemble of cells, including cancer cells,[6,7] fibroblasts,[8] endothelial cells,[9] immune cells,[10] and the extracellular matrix.[11] TNBC is characterized by significant infiltration of immune cells which is often associated with aggressive behavior and poor prognosis. Understanding how different immune cells influence the development and therapy response of TNBC is an area of active research. However, due to the lack of actionable targets, conventional therapeutic approaches for TNBC are somewhat limited. Consequently, the comprehension of TNBC progression mechanisms and identifying novel therapeutic targets remain a significant area of research and clinical interest.

Nuclear factor erythroid-2-like factor 3 (NRF3) is a member of the Cap' n' collar basic leucine zipper family of transcription factors.[12,13] The remarkable development of human cancer genome databases has strongly suggested the functional significance of NRF3 in cancer. Specifically, elevated expression NRF3 mRNA has been demonstrated across numerous cancer types, including colorectal cancer and pancreatic adenocarcinoma,[14–16] suggesting an essential role for NRF3 in cancer progression. Moreover, NRF3 has been shown to play a vital role in cancer progression-related processes such as cell proliferation, invasion, and angiogenesis.[14] Meanwhile, NRF3 is instrumental in stress-associated transcriptional programs that activate protective responses against a variety of cellular stressors, including oxidative injury and protein aggregation.[17] In the TME, NRF3 can influence the expression of genes related to inflammation and immune regulation.[18,19] Besides, NRF3 mRNA expression is found to be significantly elevated in a multitude of cancer types and has been identified as one of the most prominently mutated genes across a total of 12 different cancer types.[20] Nevertheless, the correlation between high NRF3 mRNA levels and patient survival has been described as either positive or negative depending on the specific type of cancer (https://www.proteinatlas.org/ENSG00000050344-NFE2L3/pathology). In certain instances, the NRF3 transcription factor has been identified as a tumor suppressor; for example, NRF3 was confirmed to suppress squamous carcinogenesis.[21] Furthermore, our previous work

has revealed that NRF3 inhibits BC cell invasion and proliferation, which potentially predicts a more favorable survival outcome for BC patients.[22]

The high mobility group box-1 protein (HMGB1) is a widely recognized inflammation-associated protein that exhibits dysregulation in a myriad of diseases, including arthritis, sepsis, and atherosclerosis.[23–25] Intracellularly, HMGB1 functions as a DNA chaperone due to its highly conserved nature as a chromatin-binding protein. Extracellularly, it serves as a quintessential damage-associated molecular pattern, capable of engaging with cytokines, chemokines, and growth factors.[26,27] HMGB1 has been shown to play dual roles in cancer biology; it can act as a tumor suppressor or promoter depending on the cellular context and environment. In many cancer types, including breast cancer, elevated levels of HMGB1 have been associated with poor prognosis and increased metastasis.[28] Consequently, HMGB1 emerges as a promising therapeutic candidate in oncology. NRF3 is a key regulator in multiple cancer types; however, its specific role in TNBC remains to be fully elucidated. Prior studies have demonstrated that HMGB1 exhibits elevated expression levels in TNBC and serves as a significant prognostic indicator for breast cancer progression and metastasis.[29] Meanwhile, TNBC, as the most aggressive form of BC, is still incurable and has a high tendency to metastasize. It presents an extremely challenging disease that requires further in-depth study of the complex mechanisms underlying it. This study aims to elucidate the role of NRF3 in TNBC progression and explore its potential interaction with HMGB1. By investigating these molecular pathways, we hope to identify novel therapeutic targets and improve the clinical outcomes for patients with TNBC. (a flowchart to introduce the research methods used in the study as shown in Figure S5).

2. Materials and methods

2.1. Data download and analyses

In this study, 1217 breast cancer samples normalized with $\log_2$ (count +1) expression were collected from the TCGA database (https://www.cancer.gov/ccg/research/genome-sequencing/tcga), including 1104 tumors and 113 normal tissue samples. Based on the clinical information of the samples, the study retained 122 TNBC samples that were negative for ER, PR, and HER2 expression, and 113 normal breast tissue samples for further analysis. The samples were divided into two groups based on the median expression level of NRF3, and a Kaplan-Meier (K-M) curve was used to analyze the survival of the NRF3 gene in TNBC samples. Differential analysis was performed between the high and low NRF3 expression groups, with a differential gene threshold of fdrFilter = 0.05; $\log_2$ FCfilter = 1. NRF3-related differential expressed genes (DEGs) in TNBC were used for the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), and Gene Set Enrichment Analysis (GSEA). The CIBERSORT algorithm in the "IOBR" package of R software was used for NRF3 immune infiltration analysis.

Breast cancer single-cell data was downloaded from the GSE176078 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE176078). The Seurat method was used to re-cluster and group single-cell data and the PCA method was used to reduce the dimensions of the main components. The "JackStrawPlot" and "ElbowPlot" were used to determine the selection of 1–30 dimensions for subsequent analysis of single-cell data. The cluster category adjustment was conducted using a k.param parameter of 20 and cell type identification was performed using a resolution of 0.2. Ten TNBC samples were screened. The UMAP model, based on Riemannian geometry and algebraic topology theory framework, was used to reduce dimension mapping of cells in TNBC samples. The "VlnPlot" function was used to visualize the expression of CD86 in the sample of NRF3 high and low expression.

2.2. Cells and culture

The MDA-MB-468 (CL-0290A) and Hs578t (CL-0114) cell lines of TNBC used in this study were purchased from Pricella (Wuhan, China), and were respectively cultured in Leibovitz's L-15 medium (Pricella, Wuhan, China, PM151010) or DMEM (Pricella, Wuhan, China, PM150210). Both media contained 10% fetal bovine serum (FBS; Invitrogen, CA, USA 15,070,063) and 1% Pen-strep (Procell, Wuhan, China, PB180120). The cells were incubated at 37°C with 5% CO_2 using an incubator (Thermo, MA, USA, NO. 311).

2.3. Western blotting (WB)

WB assays were performed to detect the expression of proteins. In brief, total proteins from pretreated cells were extracted, and their concentration was evaluated using a BCA Protein Assay kit (Takara, Beijing, China, T9300A). After separating the protein samples via SDS-PAGE, they were transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% BSA (Solarbio, Beijing, China, A8020), chemiluminescence was conducted utilizing ECL (Thermo, Boston, USA 20,148) following incubation with primary and secondary antibodies. Details of the antibodies used are shown in Table 1.

2.4. Real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from cells treated with Trizol reagent (Ambion, USA 15,596,018), and 100 ng of total RNA was used for reverse transcription using a cDNA synthesis kit (Takara, Beijing, China, 6210A). The obtained cDNA was then used to perform qPCR using an ABI-7500 system (Thermo Fisher, Waltham, MA, USA 4,351,106). The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and the primers are listed in Table 2.

2.5. Transient transfection

To illustrate the role of NRF3 in TNBC cells, the NRF3 stably overexpressed, and HMGB1 stably silenced Hs578t cells and the corresponding control cell were generated using lentiviruses. Briefly, 100 μL of NRF3-overexpressing (hereafter,

Table 1. Information of antibodies.

Name	Brand	Article number	Dilution rate	Applications
NRF3	Proteintech	17745-1-AP	1:500-1:1000	WB
E-cadherin	Santa Cruz	sc-8426	1:1000	WB
N-cadherin	Santa Cruz	sc -59,987	1:1000	WB
Vimentin	Abclonal	A19607	1:2000	WB
MMP2	CST	4022	1:1000	WB
MMP9	Proteintech	10375-2-AP	1:1000	WB
ERK	Proteintech	51068-1-AP	1:2000; 1:100	WB; IHC
MEK	Proteintech	11049-1-AP	1:500	WB
HMGB1	Proteintech	10829-1-AP	1:1000; 1:6000	WB; CHIP
Ki67	Santa Cruz	sc -376,764	1:1000	IHC
β-actin	CST	4970	1:5000	WB

Table 2. Information of primers.

Names	F(5′-3′)	R(5′-3′)
NRF3	TACCGTATTTATGATGGGACT	GCTGACATTCAAGGGACAC
SOD2	GGACAAACCTGAGCCCTAA	GCGACCTTGCTCCTTATTG
PrxI	TTTTACCCTCTTGACTTTAC	AATCCTCCTTGTTTCTTGG
NQO1	CAGCGGCTTTGAAGAAGA	GCAGGGGGAACTGGAATA
β-actin	AGCAGTTGTAGCTACCCGCCCA	GGCGGGCACGTTGAAGGTCT

Lenti-NRF3), HMGB1-silencing (hereafter, Lenti-sh-HMGB1) , and the control lentivirus (hereafter, Lenti-control) (1×10^8 Tu/mL) were obtained from Guannan Biological (Hangzhou, China) to transfect Hs578t cells using lipofectamine 2000 (Invitrogen, Waltham, USA 11,668–019), respectively. After 72 hours of transfection, positive cells were screened with 0.2 μg/mL puromycin (Thermo Fisher, A1113802). Subsequently, the efficiency of protein expression in stably transfected cells was confirmed by Western blotting.

For the transient overexpression of NRF3 or NQO1 and the silencing of HMGB1 MDA-MB-468 and Hs578t cell lines, the plasmids of siRNAs targeting the HMGB1 and the control siRNAs, as well as the plasmids of overexpressed NRF3 or NQO1 were provided by Hippo Bio (Hangzhou, China). The overexpressed plasmids were cloned into the pcDNA3.1 vector (Thermo Fisher, V79020). Then, MDA-MB-468 and Hs578t cells were transfected above plasmids using Lipofectamine 2000 and adding 50 μL Opti-MEM (Gibco, Grand Island, NY, USA 31,985-–070) for 24 hours, respectively. Subsequently, the efficiency of transfection was detected by Western blotting. The sequence information is listed in Table 2 and information on the siRNAs is as follows:

siR-NC-sense: UUCUCCGAACGAGUCACGUTT
siR-NC-antisense: ACGUGACUCGUUCGGAGAATT
siR-HMGB1-sense: CCGUUAUGAAAGAGAAAUGAA
siR-HMGB1-antisense: UUCAUUUCUCUUUCAUAACGG

2.6. MTT assay

The MTT assays were applied for the detection of cell proliferation in MDA-MB-468 and Hs578t cells. In short, 100 μL cell suspensions were seeded into 96-well plates at a concentration of 2×10^3 cells/well. Then, cells were cultured in an incubator for 0, 24, 48 and 72 h. The liquid supernatant was removed at the corresponding time points and 50 μL MTT reagent (1 mg/mL) (Beyotime, Shanghai, China, C0009S) was added. After incubation at 37°C for 3 h, DMSO was added to the plate (150 μL per well) for dissolving the crystals. The absorbance at 570 nm was measured using a microplate reader (Molecular Device, California, USA).

2.7. Transwell experiments

To detect the migration and invasion abilities of MDA-MB-468 and Hs578t cells, transwell experiments were performed. For the migration assay, 100 μL pretreatment cells suspension (1×10^4 cells) were seeded into the upper transwell chambers (Millipore, MA, USA, CLS3396) containing serum-free DMEM medium. The bottom compartment of the plate included DMEM with 10% fetal bovine serum (FBS). After 24 h of incubation, the migrating cells were fixed with 4% paraformaldehyde (500 μL) for 15 minutes and subsequently stained with 0.1% crystal violet (500 μL) for 30 minutes. Finally, images of cell migration were observed with the microscope (Olympus Corp, Tokyo, Japan, Olympus IX73) at × 100, and the number of migrating cells was counted. For the invasion assay, except that the transwell chamber was coated with pre-diluted matrigel (Corning, NK, USA 354,234), the remainder of the experimental procedures were consistent with the migration assay.

2.8. Colony formation assay

To examine the clone-forming ability of MDA-MB-468 and Hs578t cells after the indicated treatments, the colony formation assay was performed. Briefly, 100 μL cell suspension (5×10^3 cells/mL) was cultured in a 6-well plate with the basic medium containing 10% FBS for 2 weeks. Throughout this period, the medium was replaced with a fresh one every 3 days. Afterward, the medium was removed, and cells were fixed with 4% paraformaldehyde (1 mL) for 30 minutes, and then stained with 1% crystal violet (1 mL) for 20 minutes. Finally, cells were observed under a microscope and counted using the Image processing software (Image J).

2.9. Flow cytometry

The flow cytometry was implemented to determine cell apoptosis and the level of Reactive Oxygen Species (ROS) in MDA-MB-468 and Hs578t cells after designated pretreatment. For cell apoptosis, the procedures of detection were performed using an Annexin V-FITC/7-AAD apoptosis detection kit (Procell, Wuhan, China, p-CA-202)

according to the producer's directions. The results were examined by a flow cytometer (Invitrogen, Waltham, USA, Attune NxT).

For the level of ROS, a ROS Assay Kit (Beyotime, Shanghai, China, S0033S) with the fluorescent probe DCFH-DA was used. Briefly, cells were treated with the DCFH-DA diluted in the serum-free medium at 1:1000 for incubation. After incubation for 20 minutes, flow cytometry was used to immediately detect the levels of total ROS.

2.10. Immunofluorescence (IF)

To explore the intracellular expression of HMGB1 in the TNBC cell lines with NRF3 overexpression, the IF experiment was performed according to a previously published method.[30] The result was observed under an inverted fluorescent microscope (OLYMPUS, CKX53, Japan). Additionally, an IF assay was also performed to stain the sections of the transplanted tumor and detect the levels of CD86, a biomarker of M1 macrophages, to reveal the infiltration rate of M1 macrophages. Briefly, 5 μm paraffin sections were dewaxed and rehydrated, heated in citric acid buffer (antigen repair solution, PH = 6.0), and then sealed with 3% bovine serum albumin (Servicebio®, Wuhan, China, GC305010) for 30 minutes. A mixture of primary antibody CD86 (CST, USA, 91882T) and F4/80 (Thermo Fisher, 14-4801-82) (CD86:F4/80 = 1:1) was added for overnight incubation. After incubation for 50 minutes with anti-mouse IgG, HRP-linked antibody (CST, 7076 V), DAPI dye solution (Servicebio®, G1012) was added for 10 minutes, and autofluorescence quenching agent (Servicebio®, G1221) was added for 5 minutes. The result was observed under an inverted fluorescent microscope after the section was sealed with an antifluorescent quenching agent (Servicebio®, G1401).

2.11. Enzyme-linked immunosorbent assay (ELISA)

The ELISA assay was used to examine the content of HMGB1 in MDA-MB-468 and Hs578t cells with NRF3 overexpressed, and NRF3 overexpressed combined with ROS inhibitor or HMGB1 silenced, respectively. The experimental procedure of ELISA was conducted using an ELISA detection kit (MEIMIAN, Jiangsu, China 12,907) in line with the producer's instructions. The result of ELISA was examined using a microplate reader at the wavelength of 450 nm.

2.12. Dual-luciferase reporter

To validate the regulation relationship between NRF3 and NQO1, a Dual-luciferase reporter assay was performed. The plasmid of NQO1 wild type and its mutant were obtained from Hippo Bio (Hangzhou, China), and then were cloned into the PGL3 vectors. The above plasmids and the corresponding control plasmids were transfected into MDA-MB-468 and Hs578t cells with NRF3 overexpression or the corresponding control cells using Lipofectamine 2000 for 24 h, respectively. Finally, luciferase activity was assessed using a Dual-Luciferase® Reporter Assay kit (Promega, USA, E1910) according to the manufacturer's specifications.

2.13. Chromatin immunoprecipitation (ChIP)

According to the instructions of the CHIP-Seq High Sensitivity Kit (Abcam, Cambridge, UK, ab185908), the NRF3-overexpression MDA-MB-468 and Hs578t cells and the corresponding control cells were cross-linked and immobilized to form a protein-DNA complex. Next, the cells were collected and ultrasonically broken to obtain DNA fragments. Subsequently, immunoprecipitation was performed using antibodies and protein A/G MagPoly Beads. Then, a DNA Clean-Up Kit (Omega, Norcross, GA, USA, D6296–01) was used to purify DNA, and the enrichment degree of the target fragments was validated by qPCR. The primer sequence is listed in Table 2.

2.14. Animal experiments

Experimental procedures of animal experiments in this work were in accordance with the principles of the Guiding Principles for the Breeding and Use of Animals in China. To construct the xenograft tumor tissues, a total of 24 nude mice (4-week-old) were purchased from Slaccas (Shanghai, China) and maintained in the designated condition (pathogen-free, day/night: 12/12, 22° C) for approximately 1 week. Then, the mice were subcutaneously injected with 8×10^6 HMGB 1 stably silenced, stably NRF3-overexpression Hs578t cells with or without silenced HMGB 1 and the corresponding control cells, respectively. Tumor growth was monitored until the tumor volume reached 1500 mm^3 (long diameter × short diameter^2 ×1/2), the mice were euthanized, and the tumors were isolated from the mice and photographed. The weight of the tumor was calculated and the diameter was measured by a caliper (SYNTEK, Hangzhou, SY-119-200).

2.15. Immunohistochemistry (IHC)

The expression of Ki67 and p-ERK of the tumor tissues was determined by the IHC assay. The tumor tissue slices of HMGB 1 stably silenced, stably NRF3-overexpression Hs578t cells with or without HMGB 1 silenced, and the corresponding control were prepared to perform the IHC assay as previously reported.[31] A microscope was used to observe the results.

2.16. TUNEL assay

The cell apoptosis of the tumor tissues with designated treatment was detected with the TUNEL assay. After paraffin embedding, the tumor tissues were cut into slices and deparaffinized. Then, the subsequent experimental procedures followed by the manufacturer's directions of the TUNEL cell apoptosis detection kit (Abcam, Cambridge, UK, ab66110). A microscope was used to observe the results.

2.17. Statistical analysis

The statistical analysis of the representative data was carried out using GraphPad Prism 6.0. Statistical comparisons between two groups were assessed by t-test and that of

multiple groups were evaluated by one-way ANOVA. A p value of less than 0.05 is considered significant.

3. Results

3.1. Expression and bioinformatic analysis of NRF3 in TNBC

Current research and databases have documented a negative association between NRF3 expression and poor prognosis, indicating an essential role of NRF3 in cancer malignancy,[21] including BC. To explore the underlying mechanism of NRF3 in TNBC, we first observed its clinical information in TNBC through the TCGA database. The box plot of the normalized expression levels showed that NRF3 expression was significantly higher in TNBC samples than in normal breast tissue. (Figure 1a). There were notable differences expression of NRF3 in different stages of TNBC, with the most obvious difference in the early stage of cancer (Table 3). The subsequent statistical analysis of prognosis also indicated that the overall survival of the high NRF3 expression group was better than that of the low expression group (Figure 1b). The NRF3-related DEGs were further obtained from the TCGA database. The GO and KEGG enrichment analysis suggested that the NRF3-related DEGs were enriched in the main pathways including oxidative stress, inflammation-related pathways, and the MAPK/ERK signal pathway, and the results were further verified by the GSEA enrichment analysis (Figure 1c–e). Besides, the data indicated that high expression of NRF3 in TNBC was linked to increased levels of transcripts associated with immune cells, indicating enhanced immune surveillance functions (Figure 1f). Additionally, utilizing the GSE176078 database for single-cell sequencing data to further validate, a total of 10 TNBC samples were screened through analysis and the UMAP model was used to reduce dimension mapping of cells in TNBC samples (Figure S1 A). Three samples exhibiting high and three with low NRF3 expression were chosen, forming NRF3 high and low expression groups. Macrophages in the 10 samples were screened, and it was found that the enrichment degree of the M1 polarization marker (CD86) in macrophages with high NRF3 expression was higher than that in macrophages with low NRF3 expression. The "VlnPlot" function was utilized to visualize the CD86 expression across samples. (Figure S1 B). This indicates that macrophages in the high NRF3 expression group tend to be more in the M1 state, meaning NRF3 promotes macrophage polarization toward M1. All the data analyses showed that the NRF3 may serve as an anticancer role in TNBC.

3.2. NRF3 inhibited the malignant phenotypes of TNBC cells as a tumor suppressor

To further verify the role of NRF3 in TNBC, we first examined the expression levels of NRF3 in different TNBC cell lines, including HCC1937, Hs578t, MDA-MB-231, and MDA-MB -468. As shown in Figure 2a, the expression levels of NRF3 were found to be significantly higher in the HCC1937 and MDA-MB-231 cell lines, whereas they were notably lower in the Hs578t and MDA-MB-468 cell lines. These latter two cell lines were then utilized for further exploration of NRF3's function in the subsequent experiments. The NRF3-overexpression MDA-MD-468 and Hs578t cell lines were constructed, and the efficiency of transient overexpression was validated by WB assay (Figure 2b). Interestingly, our data showed that NRF3-overexpression significantly suppressed the proliferation, migration, invasion, and clonogenic abilities of two TNBC cell lines, while promoting cell apoptosis (Figure 2 c–f). Given that NRF3 plays a vital role in the EMT process, inflammatory response, and the cellular response to oxidative stress,[32–34] we detected the expression levels of essential protein biomarkers of EMT, which are critical for cell mobility and tumor metastasis,[35] the expression of MMP related to inflammation,[36] and the levels of ROS. Not surprisingly, our data revealed that overexpression of NRF3 effectively inhibited the process of EMT, the expression of MMPs-related proteins, and the activation of the MAPK/ERK signaling pathway, while increasing the level of ROS in MDA-MB -468 and Hs578t cell lines (Figure 3a–c). Meanwhile, NRF3 was also silenced in MDA-MB-231 cell lines and verified. As shown in Figure S2&S3, silencing NRF3 reversed the previous experimental results. All of these findings supported that NRF3 may be deeply involved in the progression of TNBC as a tumor suppressor through the process of EMT, inflammatory, and oxidative stress response.

3.3. NRF3 inhibited the progression of TNBC by regulating the oxidative stress to inactivate the MAPK/ERK signaling pathway

Given that overexpression NRF3 inactivated the MAPK/ERK signaling pathway and increased the level of ROS in MDA-MB -468 and Hs578t cell lines, we performed the rescue experiments using the N-Acetyl-L-cysteine (NAC), a ROS inhibitor, to define the underlying mechanism for NRF3 in TNBC. As shown in Figure 4 a–c, the inhibition effect of overexpression NRF3 on the malignant phenotypes of TNBC cells could be significantly reverted by ROS inhibitor, besides, its inhibition effect on the MAPK/ERK signaling pathway was also significantly impaired by applying the ROS inhibitor (Figure 4d). In addition, through the analysis of mRNA expression levels of genes related to antioxidant stress in TNBC cell lines with NRF3 overexpression, we detected a significant downregulation of NQO1 expression, which is likely due to the overexpression of NRF3. Consequently, we further examined the expression of NQO1 in NRF3-overexpressing cells and confirmed that it was indeed downregulated (Fig. S4 A). The subsequent Luciferase and ChIP assays further verified the regulatory relationship between NRF3 and NQO1 (Figure 5a, b–c). We then increased the level of NQO1 and confirmed its overexpression efficiency by WB (Fig. S4 B). More importantly, the rescue experiments confirmed that overexpression of NRF3 and NQO1 combined in TNBC cell lines significantly impaired the inhibition effect of NRF3 on cell proliferation,

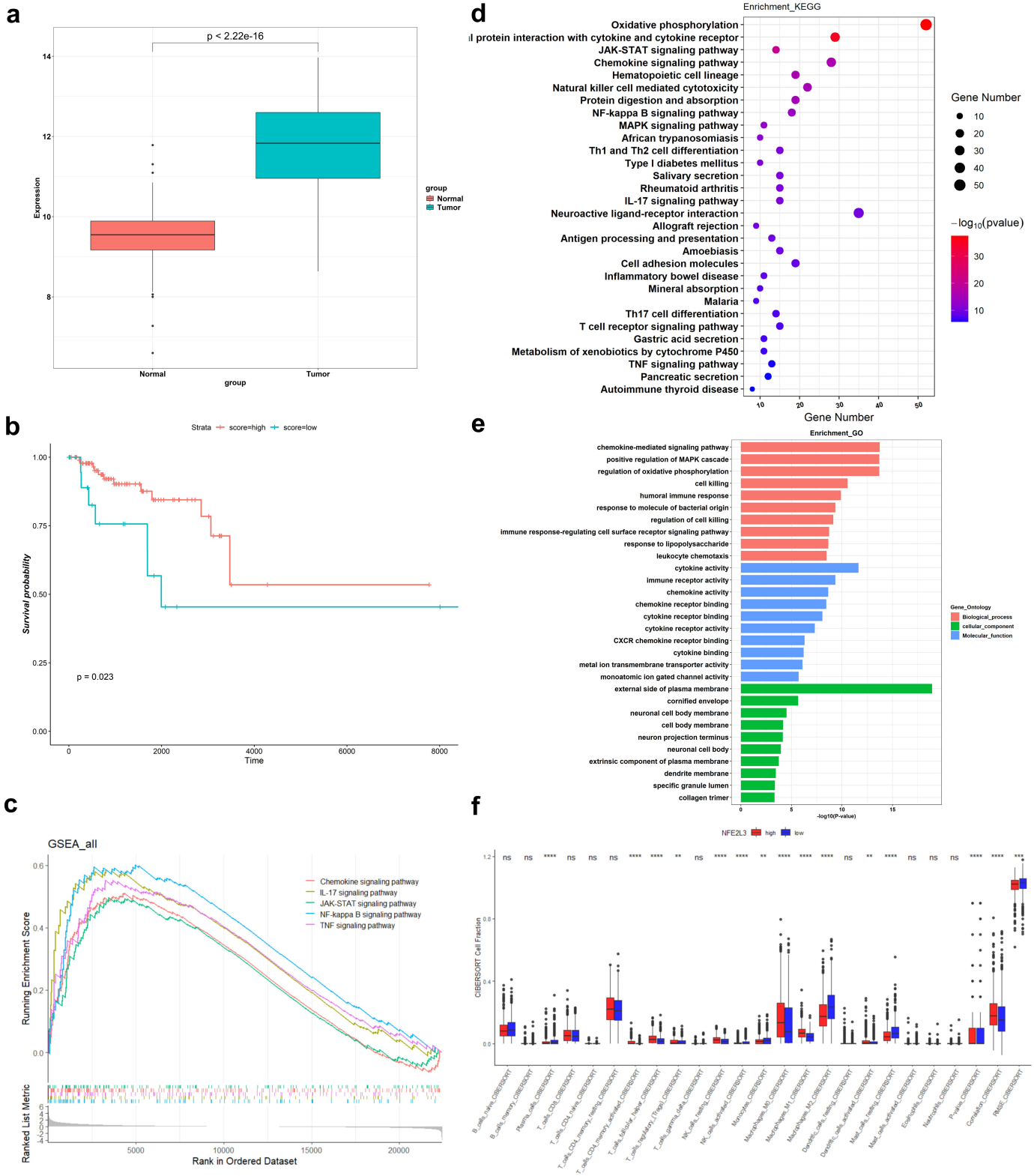


Figure 1. Expression and clinical significance of NRF3 in TNBC. TCGA database was used to analyze (a) the expression of NRF3 in the 122 TNBC samples and 113 normal breast tissue samples, as well as (b) a Kaplan-Meier (k-m) curve was used to analyze the prognostic statistical analysis of NRF3 expression in TNBC samples. GSEA (c), KEGG (d), and GO (e) were used to analyze the enrichment pathways of NRF3-related DEGs in TNBC samples. (f) The immune infiltration box plot showed the effect of NRF3 expression in TNBC on immune cells.

migration, invasion, and the activation of MAPK/ERK signaling pathways, and the promotion effect on ROS level (Figure 5d–g). Taken together, our findings proved that NRF3 was deeply involved in oxidative stress response by

Table 3. The correlation between NRF3 expression and clinical significance of TNBC.

Character	Level	Low expression of NRF3	High expression of NRF3	P
Age	≤65	271 (32.5%)	340 (40.8%)	0.859
	>;65	100 (12.0%)	122 (14.6%)	
T	T1	106 (12.8%)	128 (15.4%)	0.945
	T2	219 (26.4%)	280 (33.7%)	
	T3	29 (3.5%)	37 (4.5%)	
	T4	17 (2.0%)	15 (1.8%)	
N	N0	153 (18.7%)	238 (29.1%)	0.003
	N1	139 (17.0%)	148 (18.1%)	
	N2	51 (6.2%)	49 (6.0%)	
	N3	21 (2.6%)	20 (2.4%)	
M	M0	322 (50.3%)	305 (47.7%)	0.202
	M1	9(1.4%)	4 (0.6%)	
Stage	I	32 (5.6%)	30 (5.3%)	0.031
	II	155 (27.3%)	150 (26.5%)	
	III	82 (14.5%)	60 (10.6%)	
	IV	39 (6.9%)	19 (3.4%)	

regulating its related gene expression and ROS level to inactivate the MAPK/ERK signaling pathway, ultimately leading to the inhibition of TNBC progression, at least partially.

3.4. NRF3 promoted the release of HMGB1 via ROS to affect the polarization of macrophages thereby suppressing the progression of TNBC

High mobility group box 1 (HMGB1), a non-histone nuclear protein strongly linked to tumor progression and chemotherapy resistance, has been reported to regulate the ROS/ERK1/2/caspase-3/GSDME signaling involved in GSDME-mediated pyroptosis in neuroblastoma.[37] HMGB1 can play different roles in a variety of conditions and participate in the occurrence and development of diseases, especially in inflammatory diseases and cancer.[38] As such, we hypothesized there was a certain regulatory effect between HMGB1 and NRF3-mediated ROS in TNBC. Based on this speculation, the intracellular expression and extracellular release of HMGB1 in TNBC cell lines with NRF3 overexpression were analyzed. The IF result revealed that HMGB1 was not only expressed in the nucleus, but also the cytoplasm, and ELISA assays showed that extracellular release of HMGB1 was positively associated with NRF3 overexpression (Figure 6a,b). Given that HMGB1 exerts its anti-tumor effect through immunogenic cell death induction in BC cells,[39] the polarization of macrophages was detected, not surprisingly, macrophages were mainly polarized toward M1 after intervention with the culture supernatant of NRF3-overexpression TNBC cell lines (Figure 6c). Subsequently, we surprisingly found that the cell supernatant of those macrophages possessed a negative association with the proliferation, migration, and invasion capacities of TNBC cell lines (Figure 6d,e), indicating that NRF3 inhibited the malignant phenotype of TNBC by inducing M1 polarization of macrophages. More surprisingly, the rescue experiments confirmed that the promotion effect of NRF3 on the intracellular expression and extracellular release of HMGB1, and the M1 polarization of macrophages could be significantly reverted by ROS inhibitor or silenced HMGB1 in NRF3-overexpression TNBC cell lines (Figure 7a–c). These findings convincingly demonstrated that NRF3 suppressed the progression of TNBC through its promotion effect on the intracellular release of HMGB1 via ROS to induce the M1 polarization of macrophages.

To further validate, we constructed a xenograft model to illustrate the role of NRF3 in TNBC *in vivo*. We respectively engineered stable Hs578t cell lines with HMGB1 silencing, stable Hs578t cell lines with and without HMGB1 silencing that overexpress NRF3, and corresponding control cells. Then, WB assays were performed to detect the efficiency of protein expression in stably transfected cell lines for the follow-up experiments (Figure 8a). The data showed that NRF3 overexpression could inhibit tumor growth, which could be significantly reverted by HMGB1 silenced. This reversal was specifically evident in tumor volume and weight (Figure 8b,c). Additionally, the subsequent IHC assay further confirmed that the levels of p-ERK and Ki67 were higher in the tumor tissues of NRF3-overexpression combined with silenced HMGB1 than that the NRF3 overexpressed alone (Figure 8d), and the result from the TUNEL assay also confirmed that HMGB1 silenced could significantly impair the promotion effect on cell apoptosis of NRF3-overexpression tumor tissues (Figure 8e). Unsurprisingly IF staining was performed on the tumor sections of each group to detect the levels of CD86, a biomarker of M1 macrophages infiltration, which showed that NRF3 overexpression promoted the polarization of M1 macrophages (Figure 8f). Taken together, these findings further supported that NRF3 could inhibit the progression of TNBC by regulating the expression of HMGB1 *in vivo*.

4. Discussion

NRF3 has emerged as a promising druggable target for various diseases, including cancer.[21,40] Its role in cellular antioxidant responses and maintenance of redox homeostasis makes it an attractive candidate for therapeutic intervention in diseases characterized by dysregulated oxidative stress, such as TNBC.[41,42] Additionally, NRF3 can influence TME by suppressing inflammation and upregulating immunosuppressive markers,[40] making it a potential drug target. Targeting NRF3 could offer a novel therapeutic strategy that works in synergy

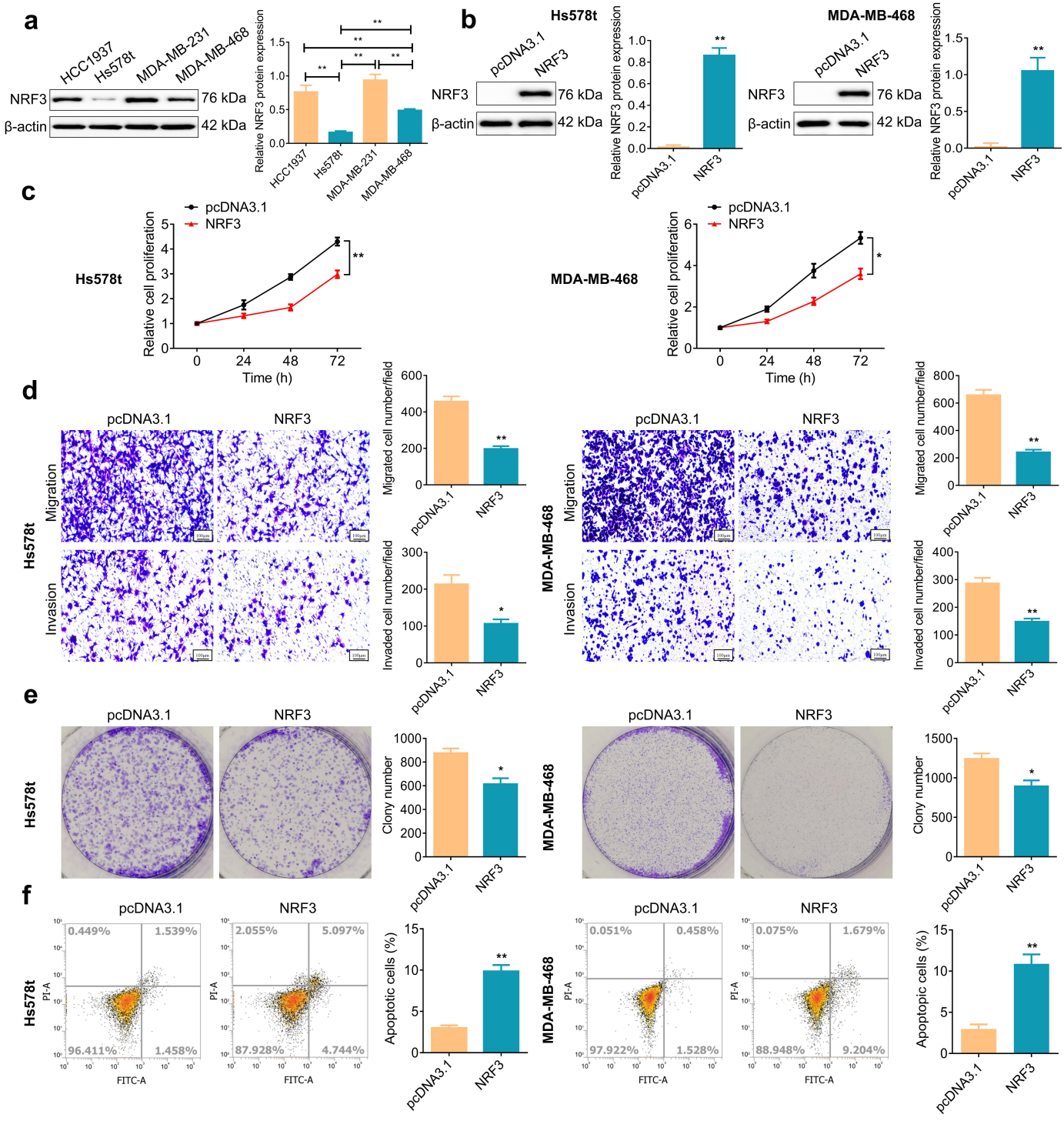


Figure 2. Effect of NRF3 overexpression on biological function of TNBC. (a) The expression levels of NRF3 in different TNBC cell lines, including HCC1937, Hs578t, MDA-MB-231, and MDA-MB-468, were detected by WB.NRF3 was overexpressed in both Hs578t and MDA-MB-468 cells. (b) WB was performed to detect the efficiency of NRF3 overexpression. (c) The MTT assay was employed to assess the cell proliferation. (d) Cell migration and invasion were evaluated using the Transwell method. (e) The colony formation assay was used to determine the clone-forming ability. (f) Cell apoptosis was analyzed by flow cytometry. Three independent experiments were performed for all assays, $*p < .05$, $**p < .01$ versus the control group. Scale bar, 100 μm.

with current treatments like chemotherapy, radiotherapy, and immunotherapy. For instance, NRF3 promotes UV-induced keratinocyte apoptosis through suppression of cell adhesion.[43] In conclusion, NRF3 holds significant promise as a druggable target in cancer treatment. However, more research is needed to fully understand its complex functions and develop effective NRF3-targeted therapies. Our previous research indicated that NRF3 overexpression could inhibit the EMT process, reduce the expression of MMPs, and suppress cell proliferation and metastasis in BC cells, suggesting that NRF3 plays a necessary role in suppressing BC metastasis.[22] We wondered whether NRF3 would exhibit the same inhibitory effect on TNBC, a unique BC subtype known for its lack of targeted treatments. In this study, we initially conducted bioanalysis to investigate

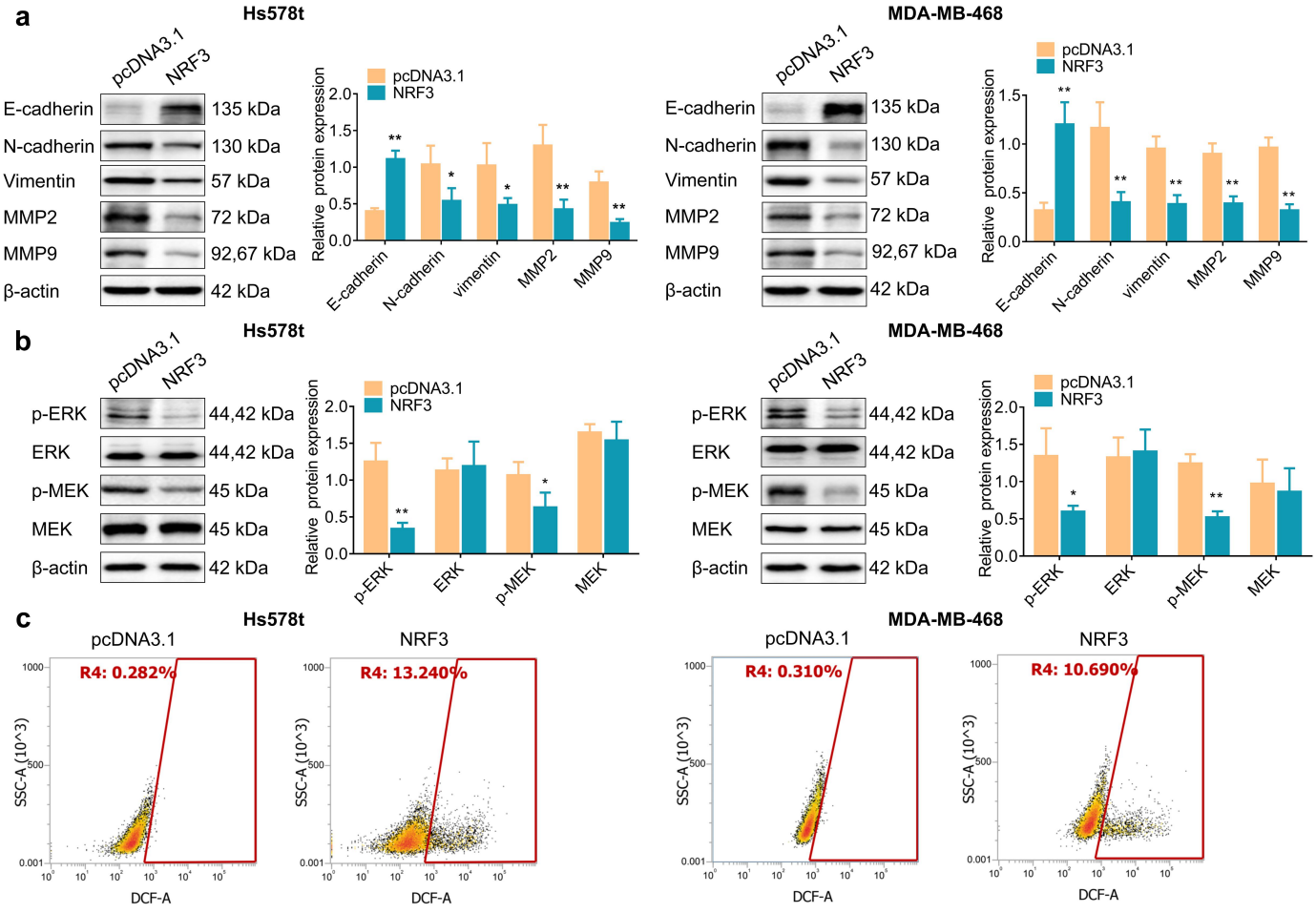


Figure 3. The regulatory effect of overexpressing NRF3 on the expression of biological function-related indicators and signaling pathways in TNBC. (a) The protein expression levels of EMT, MMP, and (b) MAPK/ERK signaling pathway biomarkers in the TNBC cell lines with NRF3 overexpression were detected by WB. (c) Flow cytometry was performed to verify the level of ROS in overexpressed NRF3 TNBC cell lines. Three independent experiments were performed for all assays, $*p < .05$, $**p < .01$ versus the control group.

the expression and clinical significance of NRF3 in TNBC. Our finding suggested that NRF3 expression correlates with a more favorable prognosis and exerts different effects on different TNBC stages. Meanwhile, our functional enrichment analysis revealed that the NRF3-related DEGs were predominantly enriched in pathways associated with oxidative stress, inflammation, and the MAPK/ERK signal pathway in TNBC. Subsequent biological function experiments showed that NRF3 overexpression inhibited the malignant phenotype of TNBC cells. However, this inhibitory effect was reversed again when NRF3 was silenced. This suggests that NRF3 is involved in the initiation and progression of TNBC as a tumor suppressor.

It has been demonstrated that EMT plays a crucial biological role in cancer progression, metastasis, and drug resistance. Similarly, ROS has been shown to act as either a tumor suppressor or a tumor promoter, depending on the tumor type and context, through its induction of EMT.[44,45] Additionally, the inflammatory response within the tumor site generates large amounts of ROS, produced by activated neutrophils and macrophages.[46] Moreover, mutations in key molecules of the MAPK/ERK pathway and its dysregulation in various human malignancies are widely recognized phenomena, and the role of ROS in regulating the activation of the MAPK/ERK signaling pathway is significant.[47] Based on this information, we performed further assays to explore the mechanisms by which NRF3 regulates the malignant phenotype in TNBC cell lines. The findings indicated that NRF3 overexpression suppresses the EMT process, reduces the expression of MMPs-related proteins, and inhibits the activation of the MAPK/ERK signaling pathway. Meanwhile, NRF3 overexpression promoted the ROS level in TNBC cell lines. Furthermore, when NRF3 was treated with NAC, a ROS inhibitor, the malignant phenotype of the cells was enhanced while the ROS level was decreased. Simultaneously, NRF3 overexpression suppressed the expression of genes involved in anti-oxidative stress (NQO1, SOD2, and prxI), suggesting NRF3 potentially regulates these genes to inhibit the activation of the MAPK/ERK signaling pathway. Rescue experiments indeed proved that NQO1 can reverse the inhibitory effects of NRF3 on the malignant phenotype of TNBC cell lines, confirming that NRF3 can suppress TNBC's biological function through regulation of oxidative stress-related genes and inhibition of the MAPK/ERK signaling pathway.

It is well-established that the levels of ROS regulate the functional activity of STAT-1, MAPKs, and NF-κB, which collectively result in the augmentation of proinflammatory signaling cascades.[48] Notably, HMGB1, a non-histone

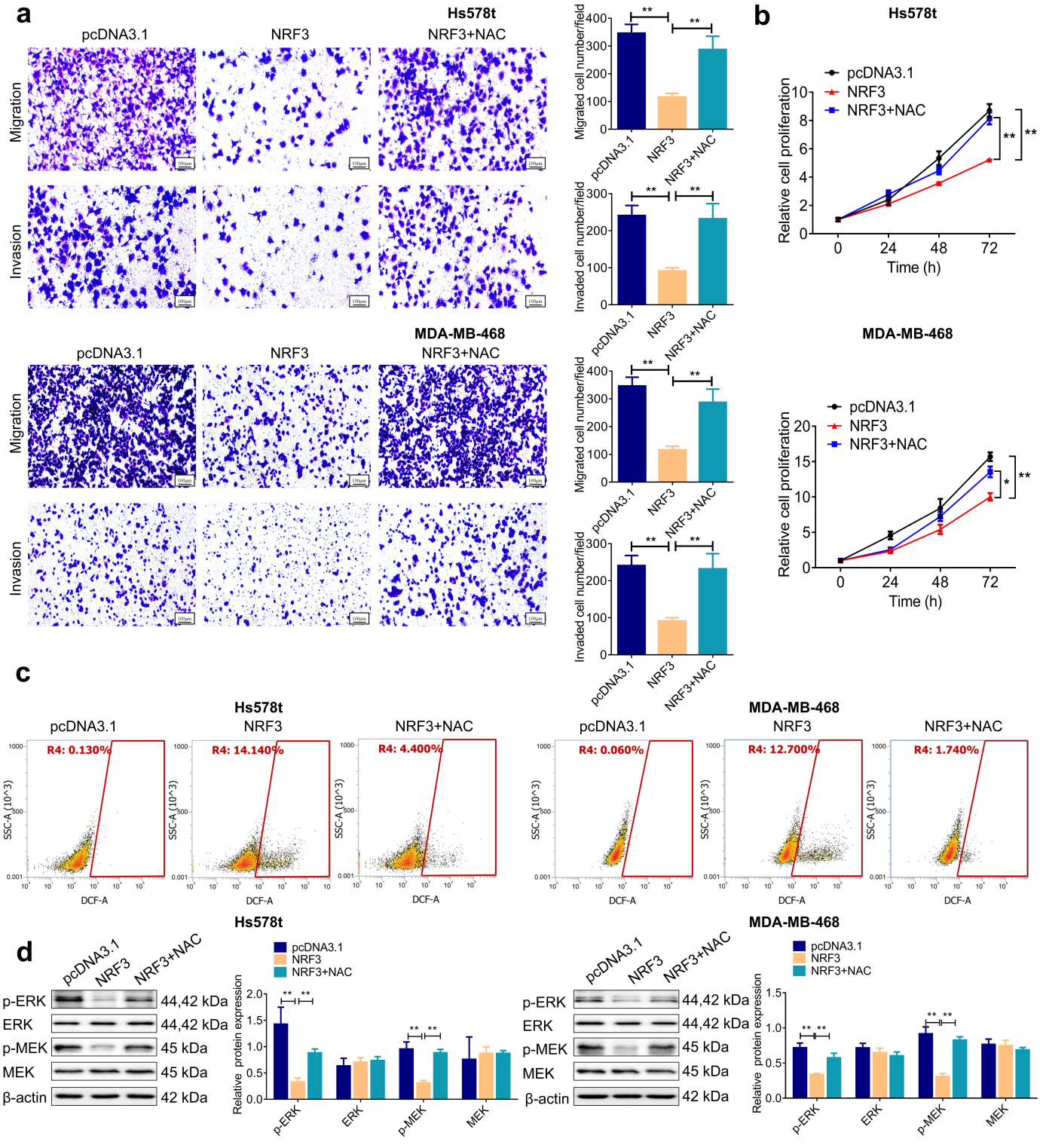


Figure 4. NRF3 inhibited the biological function of TNBC by regulating the expression of oxidative stress-related genes and inhibiting the MAPK/ERK signaling pathway. In Hs578t and MDA-MB-468 cells overexpressing NRF3, NAC was administered or not. (a)The transwell assay was utilized to examine alterations in cell invasion and migration. (b) The MTT assay was conducted to evaluate the impact on cell proliferation. (c) The level of ROS was quantified using flow cytometry. (d) WB was employed to assess the protein expression levels of MAPK/ERK signaling pathway biomarkers. Three independent experiments were performed for all assays, $*p < .05$, $**p < .01$ versus the control group. Scale bar, 100 μm.

chromatin-associated protein, has been identified as an upstream regulatory cytokine in inflammatory response.[49] Moreover, chronic inflammation-induced excessive production of HMGB1 is associated with tumorigenesis.[50] Moreover, the positive feedback release or expression of HMGB1 and RAGE through the MAPK/ERK signaling pathway in macrophages significantly promotes the polarization of M1 macrophages.[51] In this study, we found that the overexpression of NRF3 promotes the release of HMGB1 in TNBC cell lines and enhances M1 polarization of macrophages.

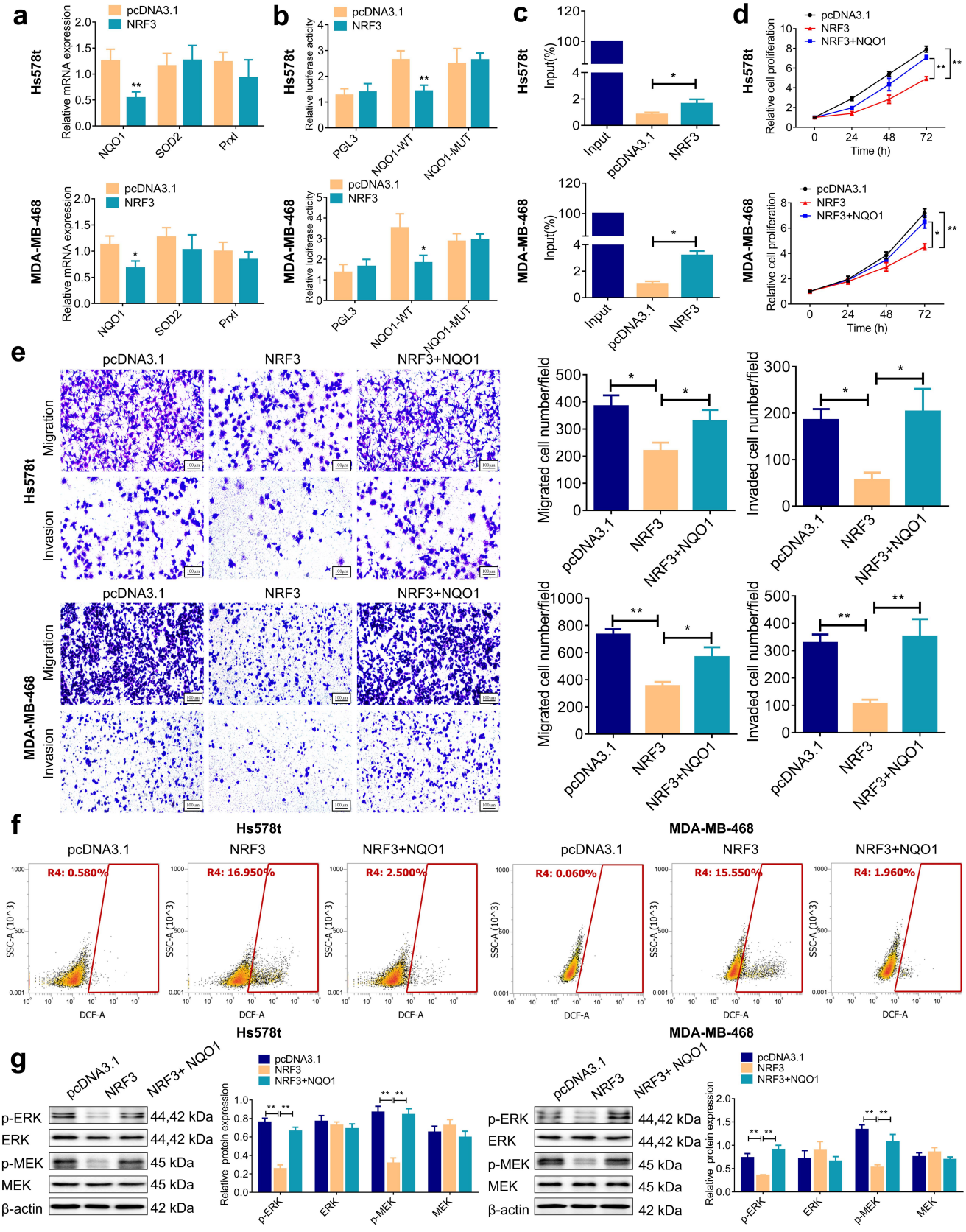


Figure 5. The inhibition effect of NRF3 on the biological function of TNBC cell lines could be significantly impaired by NQO1 expression.(a) the expression of anti-oxidative stress-related genes in Hs578t and MDA-MB-468 cell lines with NRF3 overexpression was detected by qPCR, including NQO1, SOD2, and Prxl. (b) The luciferase assay was used to validate the regulation relationship between NRF3 and NQO1 in Hs578t and MDA-MB-468 cell lines. (c) A ChIP assay was employed to detect the enrichment degree of NQO1 in the Hs578t and MDA-MB-468 cell lines with NRF3 overexpression. The Hs578t and MDA-MB-468 cell lines were engineered to overexpress either NRF3 alone or a combination of NRF3 and NQO1. (d) The MTT assay was conducted to evaluate the impact on cell proliferation. (e) The transwell assay was utilized to examine alterations in cell invasion and migration. (f) The level of ROS was quantified using flow cytometry. (g) WB was employed to assess the protein expression levels of MAPK/ERK signaling pathway biomarkers. Three independent experiments were performed for all assays, $*p < .05$, $**p < .01$ versus the control group. Scale bar, 100 μm.

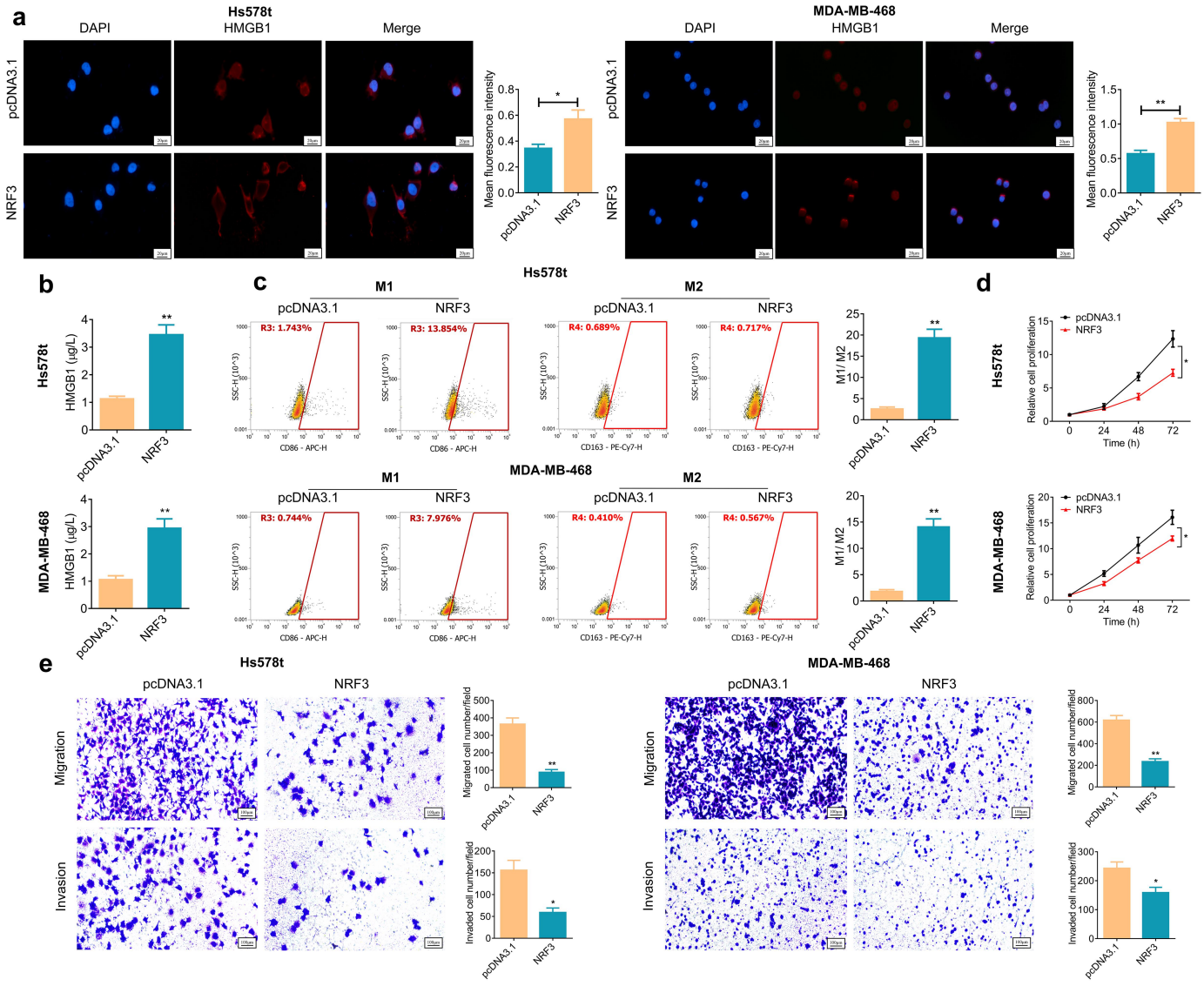


Figure 6. NRF3 promoted the release of HMGB1, thereby promoting M1 polarization of macrophages, and ultimately inhibiting the biological function of TNBC. (a) The intracellular expression and (b) extracellular release of HMGB1 in Hs578t and MDA-MB-468 cell lines with NRF3 overexpression were detected by IF and ELISA assay, respectively. Scale bar, 20 μm. (c) Flow cytometry assay was used to detect the polarization of macrophages after intervention with the culture supernatant of NRF3-overexpression TNBC cell lines. The untreated Hs578t and MDA-MB-468 cell lines were cultured in the supernatant derived from macrophages that had been induced by NRF3 overexpression. Subsequent MTT assays (d) confirmed the regulation of this macrophage culture supernatant on tumor cell proliferation, while transwell assays (e) verified its effects on cell migration and invasion. Three independent experiments were performed for all assays, $^{*}p < .05$, $^{**}p < .01$ versus the control group. Scale bar, 100 μm.

Furthermore, gain- and loss-function experiments further verify that the effects of NRF3 described above were regulated via ROS and ultimately inhibited the biological function of TNBC cell lines. The further in *vivo* assays indicated that NRF3 inhibits the growth of TNBC through HMBG1.

Studies conducted previously have extensively explored the oncogenic potential of NRF3 across various tumor types. Specifically, NRF3 overexpression has been linked to an increase in the potential for tumorigenesis within the colon and other tissues by enhancing cell proliferation and inhibiting the proteasomal degradation of tumor suppressors.[52] It is surprising that we found that NRF3 did not enhance tumorigenesis but inhibited cell proliferation, migration, invasion, and colony formation, promoting apoptosis, suggesting that NRF3 potentially serves as a tumor suppressor factor in TNBC cells. Specifically, our findings have pinpointed that NRF3 facilitates the release of HMGB1 from cells via ROS, subsequently causing the polarization of macrophages toward the M1 phenotype, which ultimately suppresses the biological functions of TNBC and curbs tumor growth, offering a promising potential therapeutic target for TNBC. Similar to our findings regarding the inhibitory effect of NRF3, several reports currently support its role as a tumor suppressor. For instance, NRF3 deficiency confers mice's susceptibility to

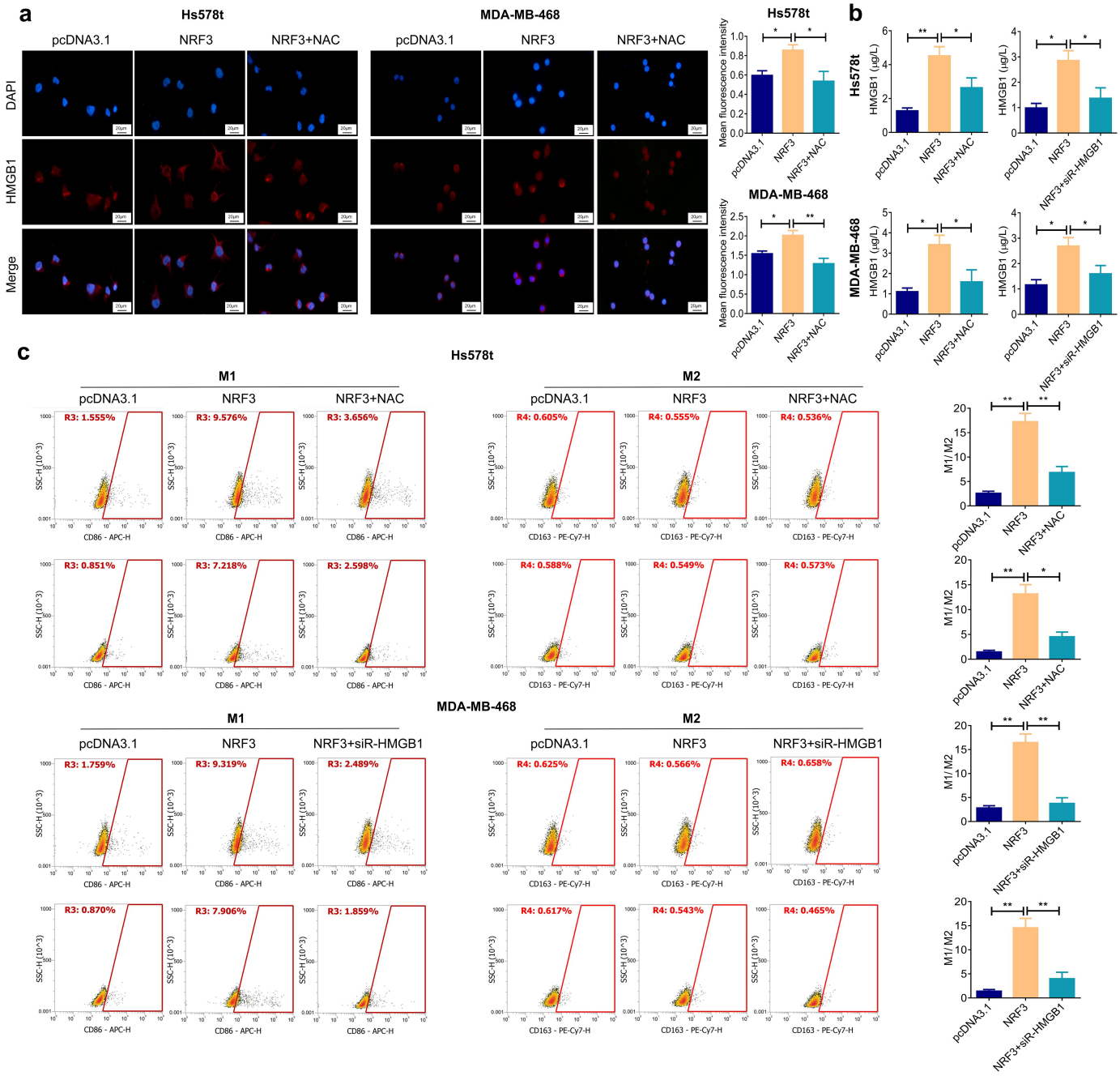


Figure 7. The promotion effect of NRF3 on the release of HMGB1 via ROS, thereby promoting M1 polarization of macrophages, could be significantly impaired by ROS inhibitor and HMGB1 silenced. IF and ELISA assays were performed to detect (a) the intracellular expression and (b) the extracellular release of HMGB1 in the overexpressed NRF3 hs578t and MDA-MB-468 cell lines combined with ROS inhibitor or HMGB1 silenced, respectively. Scale bar, 20 μm. (c) The effect on the polarization of macrophages was detected by a flow cytometry experiment in the overexpressed NRF3 hs578t and MDA-MB-468 cell lines combined with ROS inhibitor or HMGB1 silenced. Three independent experiments were performed for all assays, $*p < .05$, $**p < .01$ versus the control group.

T-cell lymphoblastic lymphoma, and the loss of NRF3 expression reduces CRC cell apoptosis and increases cell growth of CRC, thereby promoting CRC malignancy.[53] Consequently, the positive and negative effects of NRF3 in cancer progression may be influenced by the tissue the tumor stage, and the oncogenic stimulus, which could be further explored in the future. In addition, we have initially explored the underlying regulatory mechanisms of NRF3 on the malignant suppression of TNBC cell lines. However, the specific relationship between ROS, HMGB1, and MAPK/ERK signaling pathways requires more elucidation. Future studies should also aim to validate these findings in more clinically relevant models to gain insight into the mechanism of NRF3 in tumor biology and to find novel therapeutic targets.

5. Conclusions

This work unveiled a novel mechanism for NRF3 functions and the regulatory mechanism of NRF3 in TNBC cell lines. Mechanistically, NRF3 enhanced the release of HMGB1 via

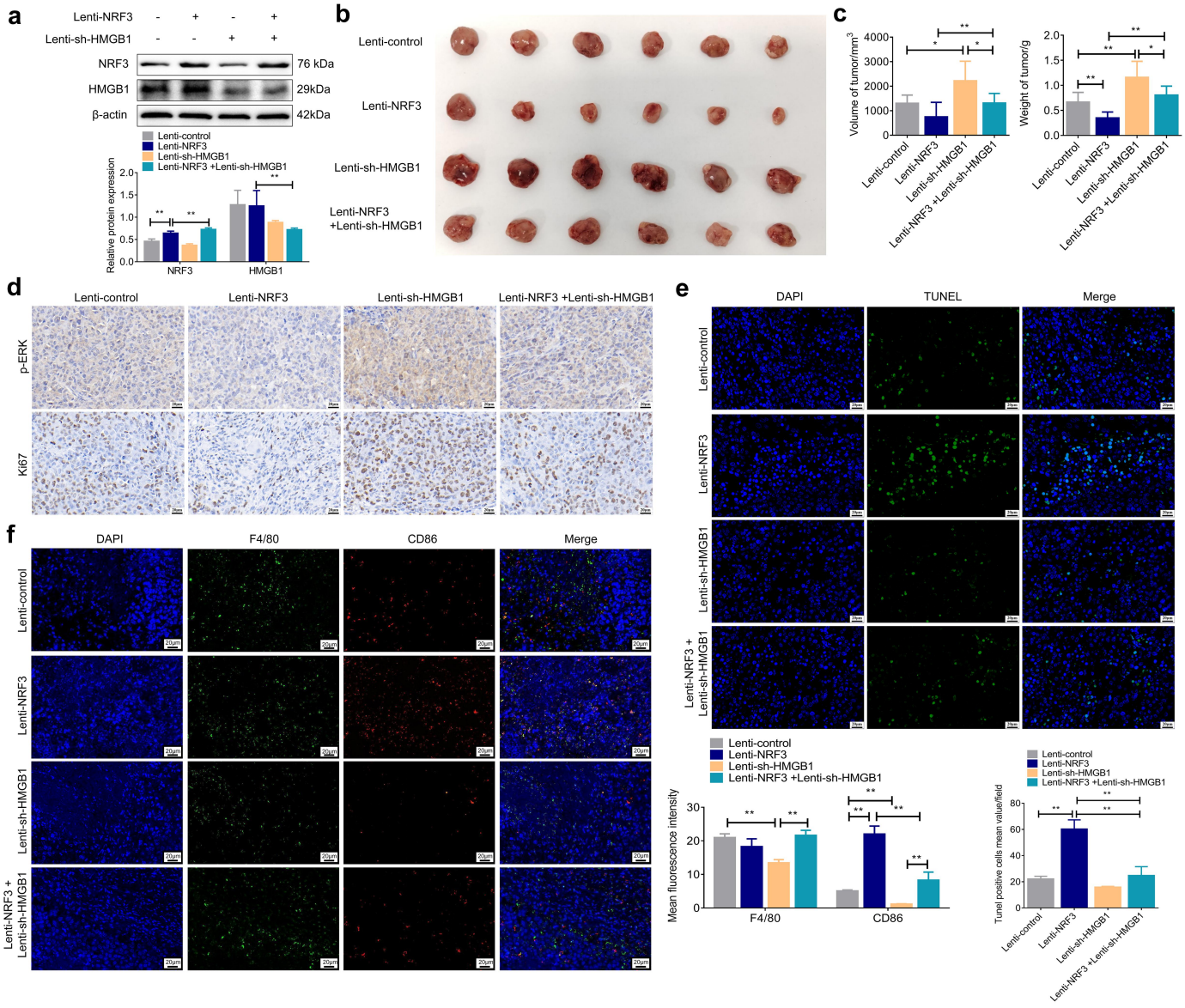


Figure 8. NRF3 inhibited the growth of TNBC through HMBG1 *in vivo*. The lentiviruses were used to generate the NRF3 alone overexpressed, HMGB1 alone silently expressed stable Hs578t cell line and their co-expression Hs578t cell line. The designated Hs578t cells was performed to construct a xenograft model using nude mice. (a) WB assay was used to identify the protein expression of stably transfected cell lines ($n = 3$). (b)&;(c) the xenograft tumors were isolated from the nude mice. The volume and weight of the tumors were recorded continuously ($n = 6$). (d) The IHC assays were used to confirm the p-ERK and Ki67 expression levels in tumor tissues ($n = 3$). Scale bar, 20 μm. (e) The TUNEL assay was used to examine the expression of apoptosis in tumor tissues ($n = 3$). Scale bar, 20 μm. (f) IF was used to detect the infiltration ratio of M1 macrophages in tumor tissues ($n = 3$). Scale bar, 20 μm. *$p < .05$, **$p < .01$ versus the control group.

ROS thus activating the M1 polarization of macrophages, ultimately leading to inhibition of the progression of TNBC.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Program of Taizhou Science and Technology Bureau, Zhejiang Province (20ywa26). Taizhou Municipal Science and Technology Bureau [20ywa26]

ORCID

Mingming Quan http://orcid.org/0009-0000-4411-0920

CRediT author contribution statement

Ping Xing: Conceptualization; Data curation; Investigation; Methodology; Writing–original draft. **Zhenzhen Chen**: Conceptualization; Data curation; Investigation. **Wenbo Zhu**: Formal analysis; Methodology. **Bangyi Lin**: Data curation; Methodology. **Mingming Quan**: Conceptualization; Investigation; Methodology; Writing–review and editing.

Data availability statement

All data generated or analyzed during this study are shown within this article.

Ethical approval statement

The animal experiments were conducted in accordance with the "Guiding Principles in the Care and Use of Animals" (China) and approved by the Laboratory Animal Ethics Committee of Taizhou Central Hospital.

References

1. Waks AG, Winer EP. Breast cancer treatment: a review. JAMA. 2019;321(3):288–300. doi:10.1001/jama.2018.19323.
2. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA: a Cancer J For Clinicians. 2023;73(1):17–48. doi:10.3322/caac.21763.
3. Pal SK, Childs BH, Pegram M. Triple negative breast cancer: unmet medical needs. Breast Cancer Res And Treat. 2010;125(3):627–636. doi:10.1007/s10549-010-1293-1.
4. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. Clin Med Res. 2009;7(1–-2):4–13. doi:10.3121/cmr.2008.825.
5. Zagami P, Carey LA. Triple negative breast cancer: pitfalls and progress. Npj Breast Cancer. 2022;8(1). doi:10.1038/s41523-022-00468-0.
6. Hu Y, He Y, Luo N, Li X, Guo L, Zhang K. A feedback loop between lncRNA MALAT1 and DNMT1 promotes triple-negative breast cancer stemness and tumorigenesis. Cancer Biol & Ther. 2023;24(1):2235768. doi:10.1080/15384047.2023.2235768.
7. Liu C, Zhang Y, Gao J, Zhang Q, Sun L, Ma Q, Qiao X, Li X, Liu J, Bu J, et al. A highly potent small-molecule antagonist of exportin-1 selectively eliminates CD44(+)CD24(-) enriched breast cancer stem-like cells. Drug resistance updates: reviews and commentaries in antimicrobial and anticancer chemotherapy. Drug Resist Updates. 2023;66:100903. doi:10.1016/j.drup.2022.100903.
8. Costa A, Kieffer Y, Scholer-Dahirel A, Pelon F, Bourachot B, Cardon M, Sirven P, Magagna I, Fuhrmann L, Bernard C, et al. Fibroblast heterogeneity and immunosuppressive environment in human breast cancer. Cancer Cell. 2018;33(3):463–479.e410. doi:10.1016/j.ccell.2018.01.011.
9. Zhou YT, Yu YQ, Yang H, Yang H, Huo YF, Huang Y, Tian X-X, Fang W-G. Extracellular ATP promotes angiogenesis and adhesion of TNBC cells to endothelial cells via upregulation of CTGF. Cancer Sci. 2022;113(7):2457–2471. doi:10.1111/cas.15375.
10. Virassamy B, Caramia F, Savas P, Sant S, Wang J, Christo SN, Byrne A, Clarke K, Brown E, Teo ZL, et al. Intratumoral CD8+ T cells with a tissue-resident memory phenotype mediate local immunity and immune checkpoint responses in breast cancer. Cancer Cell. 2023;41(3):585–601.e588. doi:10.1016/j.ccell.2023.01.004.
11. Goggins E, Mironchik Y, Kakkad S, Jacob D, Wildes F, Bhujwalla ZM, et al. Reprogramming of VEGF-mediated extracellular matrix changes through autocrine signaling. Cancer Biol & Ther. 2023;24(1):2184145. doi:10.1080/15384047.2023.2184145.
12. Kobayashi A, Ito E, Toki T, Kogame K, Takahashi S, Igarashi K, Hayashi N, Yamamoto M. Molecular cloning and functional characterization of a new cap'n' collar family transcription factor Nrf3 *. J Biol Chem. 1999;274(10):6443–6452. doi:10.1074/jbc.274.10.6443.
13. Immonen A, Haapasaari K-M, Skarp S, Karihtala P, Teppo H-R. NRF3 decreases during melanoma carcinogenesis and is an Independent prognostic Marker in melanoma. Oxidative Med And Cellular Longevity. 2022;2022:2240223. doi:10.1155/2022/2240223.
14. Chowdhury AMMA, Katoh H, Hatanaka A, Iwanari H, Nakamura N, Hamakubo T, Natsume T, Waku T, Kobayashi A. Multiple regulatory mechanisms of the biological function of NRF3 (NFE2L3) control cancer cell proliferation. Sci Rep. 2017;7(1):12494. doi:10.1038/s41598-017-12675-y.
15. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012;2(5):401–404. doi:10.1158/2159-8290.CD-12-0095.
16. Aono S, Hatanaka A, Hatanaka A, Gao Y, Hippo Y, Taketo MM, Waku T, Kobayashi A. β-Catenin/TCF4 complex-mediated induction of the NRF3 (NFE2L3) gene in cancer cells. Int J Mol Sci. 2019;20(13):3344. doi:10.3390/ijms20133344.
17. Ibrahim L, Mesgarzadeh J, Xu I, Powers ET, Wiseman RL, Bollong MJ. Defining the functional targets of Cap'n'collar transcription factors NRF1, NRF2, and NRF3. Antioxidants. 2020;9(10):1025. doi:10.3390/antiox9101025.
18. Chevillard G, Paquet M, Blank V. Nfe2l3 (Nrf3) deficiency predisposes mice to T-cell lymphoblastic lymphoma. Blood. 2011;117(6):2005–2008. doi:10.1182/blood-2010-02-271460.
19. Zhang Q, Tang D, Zha A, He J, Li D, Chen Y, Cai W, Dai J, Luan S, Yin L, et al. NFE2L3 as a potential functional gene regulating immune microenvironment in human kidney cancer. Biomed Res Int. 2022;2022:1–17. doi:10.1155/2022/9085186.
20. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, Xie M, Zhang Q, McMichael JF, Wyczalkowski MA, et al. Mutational landscape and significance across 12 major cancer types. Nature. 2013;502(7471):333–339. doi:10.1038/nature12634.
21. Gurri S, Siegenthaler B, Cangkrama M, Restivo G, Huber M, Saliba J, Dummer R, Blank V, Hohl D, Werner S, et al. NRF3 suppresses squamous carcinogenesis, involving the unfolded protein response regulator HSPA5. EMBO Mol Med. 2023;15(11):e17761. doi:10.15252/emmm.202317761.
22. Sun J, Zheng Z, Chen Q, Pan Y, Lu H, Zhang H, Yu Y, Dai Y. NRF3 suppresses breast cancer cell metastasis and cell proliferation and is a favorable predictor of survival in breast cancer. OncoTargets And Ther. 2019;12:3019–3030. doi:10.2147/OTT.S197409.
23. Wang H, Yang H, Tracey KJ. Extracellular role of HMGB1 in inflammation and sepsis. J Intern Med. 2004;255(3):320–331. doi:10.1111/j.1365-2796.2003.01302.x.
24. Andersson U, Tracey KJ. HMGB1 as a mediator of necrosis-induced inflammation and a therapeutic target in arthritis. Rheumatic Dis Clinics Of North Am. 2004;30(3):627–637, xi. 10.1016/j.rdc.2004.04.007.
25. Zhang X, Fernández-Hernando C. Endothelial HMGB1 (High-mobility group Box 1) regulation of LDL (low-density lipoprotein) transcytosis: a novel mechanism of intracellular HMGB1 in atherosclerosis. Arterioscler, Thromb, And Vasc Biol. 2021;41(1):217–219. doi:10.1161/ATVBAHA.120.315517.
26. Chen G, Ward MF, Sama AE, Wang H. Extracellular HMGB1 as a proinflammatory cytokine. J Interferon & Cytokine Res: the Off J Int Soc For Interferon And Cytokine Res. 2004;24(6):329–333. doi:10.1089/107999004323142187.
27. Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ. HMGB1 as a DNA-binding cytokine. J Leukocyte Biol. 2002;72(6):1084–1091. doi:10.1189/jlb.72.6.1084.
28. Wang Z, Yang C, Li L, Jin X, Zhang Z, Zheng H, Pan J, Shi L, Jiang Z, Su K, et al. Tumor-derived HMGB1 induces CD62L(dim) neutrophil polarization and promotes lung metastasis in triple-negative breast cancer. Oncogenesis. 2020;9(9):82. doi:10.1038/s41389-020-00267-x.
29. Huang BF, Tzeng HE, Chen PC, Wang CQ, Su CM, Wang Y, Hu G-N, Zhao Y-M, Wang Q, Tang C-H, et al. HMGB1 genetic polymorphisms are biomarkers for the development and progression of breast cancer. Int J Med Sci. 2018;15(6):580–586. doi:10.7150/ijms.23462.
30. Im K, Mareninov S, Diaz MFP, Yong WH. An introduction to performing immunofluorescence staining. Methods Mol Biol. 2019;1897:299–311. doi:10.1007/978-1-439-8935-5_26.
31. Hong S, Cho YW, Yu LR, Yu H, Veenstra TD, Ge K. Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci USA. 2007;104(47):18439–18444. doi:10.1073/pnas.0707292104.
32. Pellegrini GG, Cregor M, McAndrews K, Morales CC, McCabe LD, McCabe GP, Peacock M, Burr D, Weaver C, Bellido T, et al. Nrf2 regulates mass accrual and the antioxidant endogenous response in

bone differently depending on the sex and age. PLoS One. 2017;12(2): e0171161. doi:10.1371/journal.pone.0171161.
33. Yu MM, Feng YH, Zheng L, Zhang J, Luo GH. Short hairpin RNA-mediated knockdown of nuclear factor erythroid 2-like 3 exhibits tumor-suppressing effects in hepatocellular carcinoma cells. World J Gastroenterol. 2019;25(10):1210–1223. doi:10.3748/wjg.v25.i10.1210.
34. Chevillard G, Blank V. NFE2L3 (NRF3): the Cinderella of the Cap'n'Collar transcription factors. Cellular And Mol Life Sci. 2011;68(20):3337–3348. doi:10.1007/s00018-011-0747-x.
35. Xu H, Fu X, Liu B, Weng S, Guo C, Quan L, Liu L, Wang L, Xing Z, Cheng Q, et al. Immune perturbation network identifies an EMT subtype with chromosomal instability and tumor immune-desert microenvironment. iScience. 2023;26(10):107871. doi:10.1016/j.isci.2023.107871.
36. Medeiros NI, Gomes JAS, Correa-Oliveira R. Synergic and antagonistic relationship between MMP-2 and MMP-9 with fibrosis and inflammation in Chagas' cardiomyopathy. Parasite Immunol. 2017;39(8). doi:10.1111/pim.12446.
37. Fan CY, Ye FH, Peng M, Dong JJ, Chai WW, Deng WJ, Zhang H, Yang L-C. Endogenous HMGB1 regulates gsdme-mediated pyroptosis via ROS/ERK1/2/caspase-3/GSDME signaling in neuroblastoma. Am J Cancer Res. 2023;13(2):436–451.
38. Wang S, Zhang Y. HMGB1 in inflammation and cancer. J Hematol Oncol. 2020;13(1):116. doi:10.1186/s13045-020-00950-x.
39. Calvillo-Rodríguez KM, Mendoza-Reveles R, Gómez-Morales L, Uscanga-Palomeque AC, Karoyan P, Martínez-Torres AC, Rodríguez-Padilla C. PKHB1, a thrombospondin-1 peptide mimic, induces anti-tumor effect through immunogenic cell death induction in breast cancer cells. Oncoimmunology. 2022;11(1):2054305. doi:10.1080/2162402X.2022.2054305.
40. Saliba J, Coutaud B, Makhani K, Epstein Roth N, Jackson J, Park JY, Gagnon N, Costa P, Jeyakumar T, Bury M, et al. Loss of NFE2L3 protects against inflammation-induced colorectal cancer through modulation of the tumor microenvironment. Oncogene. 2022;41(11):1563–1575. doi:10.1038/s41388-022-02192-2.
41. Sankaranarayanan K, Jaiswal AK. Nrf3 negatively regulates antioxidant-response element-mediated expression and antioxidant induction of NAD(P)H: quinone oxidoreductase1 gene. The J Biol Chem. 2004;279(49):50810–50817. doi:10.1074/jbc.M404984200.
42. Zheng C, Pan Y, Lin B, Li J, Chen Q, Zheng Z. NRF3 suppresses the metastasis of triple-negative breast cancer cells by inhibiting ERK activation in a ros-dependent manner. Histol And Histopathol. 2024ub ahead of print Jun 26. doi: 10.14670/hh-18-786:18786.
43. Siegenthaler B, Defila C, Muzumdar S, Beer HD, Meyer M, Tanner S, Bloch W, Blank V, Schäfer M, Werner S, et al. Nrf3 promotes UV-induced keratinocyte apoptosis through suppression of cell adhesion. Cell Death Differ. 2018;25(10):1749–1765. doi:10.1038/s41418-018-0074-y.
44. Mashouri L, Yousefi H, Aref AR, Ahadi AM, Molaei F, Alahari SK. Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. Mol Cancer. 2019;18(1):75. doi:10.1186/s12943-019-0991-5.
45. Shi D, Guo L, Sun X, Shang M, Meng D, Zhou X, Liu X, Zhao Y, Li J. UTMD inhibit EMT of breast cancer through the ROS/miR-200c/ZEB1 axis. Sci Rep. 2020;10(1):6657. doi:10.1038/s41598-020-63653-w.
46. Rahat MA, Shakya J. Parallel aspects of the microenvironment in cancer and autoimmune disease. Mediators Of Inflammation. 2016;2016:4375120. doi:10.1155/2016/4375120.
47. Su X, Shen Z, Yang Q, Sui F, Pu J, Ma J, Ma S, Yao D, Ji M, Hou P, et al. Vitamin C kills thyroid cancer cells through ros-dependent inhibition of MAPK/ERK and PI3K/AKT pathways via distinct mechanisms. Theranostics. 2019;9(15):4461–4473. doi:10.7150/thno.35219.
48. Forrester SJ, Kikuchi DS, Hernandes MS, Xu Q, Griendling KK. Reactive oxygen species in metabolic and inflammatory signaling. Circ Res. 2018;122(6):877–902. doi:10.1161/CIRCRESAHA.117.311401.
49. Feng WQ, Zhang YC, Xu ZQ, Yu SY, Huo JT, Tuersun A, Zheng MH, Zhao JK, Zong YP, Lu AG. IL-17A-mediated mitochondrial dysfunction induces pyroptosis in colorectal cancer cells and promotes CD8 + T-cell tumour infiltration. J Transl Med. 2023;21(1):335. doi:10.1186/s12967-023-04187-3.
50. Yang H, Wang H, Andersson U. Targeting inflammation driven by HMGB1. Front Immunol. 2020;11:484. doi:10.3389/fimmu.2020.00484.
51. He C, Sun S, Zhang Y, Xie F, Li S. The role of irreversible electroporation in promoting M1 macrophage polarization via regulating the HMGB1-RAGE-MAPK axis in pancreatic cancer. Oncoimmunology. 2021;10(1):1897295. doi:10.1080/2162402X.2021.1897295.
52. Kobayashi A. Roles of NRF3 in the hallmarks of cancer: proteasomal inactivation of tumor suppressors. Cancers. 2020;12(9):2681. doi:10.3390/cancers12092681.
53. Zhou S, Li Y, Lu J, Chen C, Wang W, Wang L, Zhang Z, Dong Z, Tang F. Nuclear factor-erythroid 2-related factor 3 (NRF3) is low expressed in colorectal cancer and its down-regulation promotes colorectal cancer malignance through activating EGFR and p38/MAPK. Am J Cancer Res. 2019;9(3):511–528.