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NRF1 Regulates the Epithelial Mesenchymal Transition of Breast Cancer by Modulating ROS Homeostasis

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Abstract

Introduction: Nuclear respiratory factor 1 (NRF1) is an important regulator involved in mitochondrial biogenesis and energy metabolism. However, the specific mechanism of NRF1 in anoikis and epithelial-mesenchymal transition (EMT) remains unclear. **Methods:** We examined the effect of NRF1 on mitochondria and identified the specific mechanism through transcriptome sequencing, and explored the relationships among NRF1, anoikis, and EMT. **Results:** We found that upregulated NRF1 expression led to increased mitochondrial oxidative phosphorylation (OXPHOS) and ATP generation. Simultaneously, a significant amount of ROS is generated during OXPHOS. Alternatively, NRF1 upregulates the expression of ROS-scavenging enzymes, allowing tumor cells to maintain low ROS levels and promoting anoikis resistance and EMT. We also found that exogenous ROS was maintained at a low level by NRF1 in breast cancer cells. **Conclusion:** our study provides mechanistic insight into the function of NRF1 in breast cancer, indicating that NRF1 may serve as a therapeutic target for breast cancer treatment.

Keywords

NRF1, breast cancer, EMT, anoikis, OXPHOS, ROS

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Introduction

Breast cancer is a common malignant tumor and the leading cause of cancer-related death among women worldwide.[1] Metabolic reprogramming is a hallmark of cancer and cell transformation, especially in breast cancer.[2]

Breast cancer cells exhibit extensive metabolic heterogeneity.[3] Metastatic and nonmetastatic breast cancer cells show differential expression of genes responsible for mitochondrial metabolism.[4] Nonmetastatic breast cancer cells preferentially meet their energy demand through glycolysis.[5] However, invasive metastatic breast cancer cells specifically favor mitochondrial respiration, to increase ATP levels, through a mechanism that involves overexpression of PGC-1α and increases mitochondrial biogenesis.[6] Mitochondrial metabolism is still crucial for promoting cancer cell survival[7,8] and tumor adaptation to an unfavorable microenvironment.[9] Therefore, mitochondrial metabolism represents an attractive target for anti-metastatic approaches.

Anoikis is programmed cell death induced by loss of anchorage, which is important for tissue development and organ formation.[10] Anoikis resistance is a critical mechanism in tumor metastasis. Tumor cells can overcome anoikis, enabling them to proliferate and metastasize. Our previous study found that nuclear respiratory factor (NRF1) stimulated spheroid survival and activated mesenchymal traits in the nontumorigenic MCF10A and breast cancer cells.[11] Recent studies also found that NRF1 can affect multiple signaling pathways in breast cancer, such as apoptosis, cell cycle, chromosomal integrity, and DNA damage or repair.[12,13] However, the specific molecular mechanism underlying anoikis resistance and epithelial-mesenchymal transition (EMT) induced by inducing the expression of NRF1 is still unclear. Our study found that upregulated

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NRF1 expression can significantly promote ATP generation, improve the mitochondrial membrane potential, and regulate anoikis and the appearance of EMT through ROS. NRF1 may also serve as a potential target for tumor metabolic therapy.

Materials and Methods

Reagents and Antibodies

Insulin, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Madison, WI, USA). The horse serum were purchased from Invitrogen (Grand 3 Island, NY, USA). The antibodies against following protein were purchased from Cell Signaling Technology (Beverly, MA, USA): NRF1, ACTB (beta-actin), N-Cadherin (CDH2), E-Cadhein (CDH1), Vinentin (VIM), Snail (SNAI1), and ZO-1 (TJP1). The goat anti-rabbit or anti-mouse HRP-conjugated secondary antibodies were also purchased from Cell Signaling Technology (Beverly, MA, USA).

Plasmid Construction, Viral Production, and Infection

Lentiviral vector construction, viral production, and infection were carried out as described previously.[11] In brief, construction of knockdown and overexpression plasmids, generation of retrovirus, transfection of M231 cells and M10A, and 7-day drug screening. Western blotting was performed to detect the efficiency of gene expression. The shRNA for NRF1 knockdown (Sigma-Aldrich, St. Louis, MO, USA. NM_005011.2-358s1c1; TRCN0000016903; sequence: 5'-CCGGCCTCATGTATTTGAGTCTAATCTCGAGATTAGACTCAAATACATGAGGTTTTT-3').

Cell Culture

MCF10A cells (ATCC, Manassas, VA, USA) and derivative cells were cultured in defined growth medium (Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, and 1% penicillin/streptomycin). MDA-MB-231 cells (ATCC, Manassas, VA, USA) were cultured in complete DMEM containing 10% FBS, 1% penicillin/streptomycin under humidified conditions in 5% CO_2 at 37°C.

Spheroid Formation Assay

MCF10A, MDA-MB-231, and its derived cells were plated at a density of 5×10^4 per well in an ultra-low-attachment 24-well plate (Corning Inc, Lowell, USA). Cell spheroids were collected, fixed in 4% paraformaldehyde, and stained with 1% crystal violet for 10 minutes,[14] spheroids were pipetted onto glass slides and coated with cover glass on the solution. The

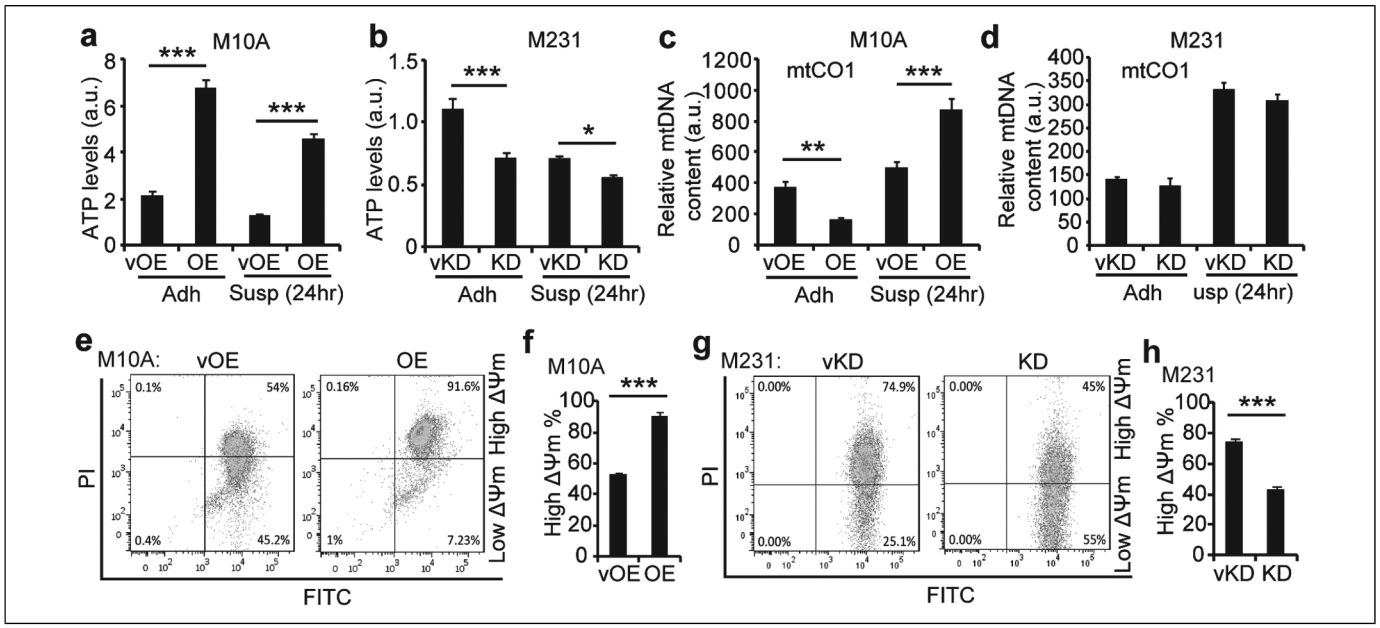

Figure 1. Upregulated NRF1 expression promotes mitochondrial biosynthesis, ATP generation, and enhances mitochondrial membrane potential. (a) The levels of ATP in NRF1-OE, and vOE M10A cells. Data was presented as mean ± SD, n = 3. (b) The levels of ATP in NRF1-KD and vKD M231 cells. Data was presented as mean ± SD, n = 3. (c) Mitochondrial DNA content in NRF1-OE and vOE M10A cells. Data was presented as mean ± SD, n = 3. (d) Mitochondrial DNA content in NRF1-KD and vKD M231 cells. Data was presented as mean ± SD, n = 3. (e-g) The levels of Mitochondrial membrane potential in NRF1-OE and vOE M10A cells. Data was presented as mean ± SD, n = 3. (g-h) The levels of Mitochondrial membrane potential in NRF1-KD and vKD M231 cells. Data was presented as mean ± SD, n = 3. *P < .05, **P < .01, and ***P < .001.

Abbreviations: NRF1, nuclear respiratory factor 1.

slides were scanned using a panoramic slide scanner (Olympus SLIDEVIEW VS200, Nanjing). The diameters of single-cell spheres were measured using the ImageJ software and the number of spheres with a diameter ≥50 μm was counted from each cell.

Mitochondrial DNA Content Measurement

Mitochondrial DNA content was measured as previously described,[11] In brief, total DNA was isolated from cells using the Genomic DNA Mini Preparation kit with Spin Columns

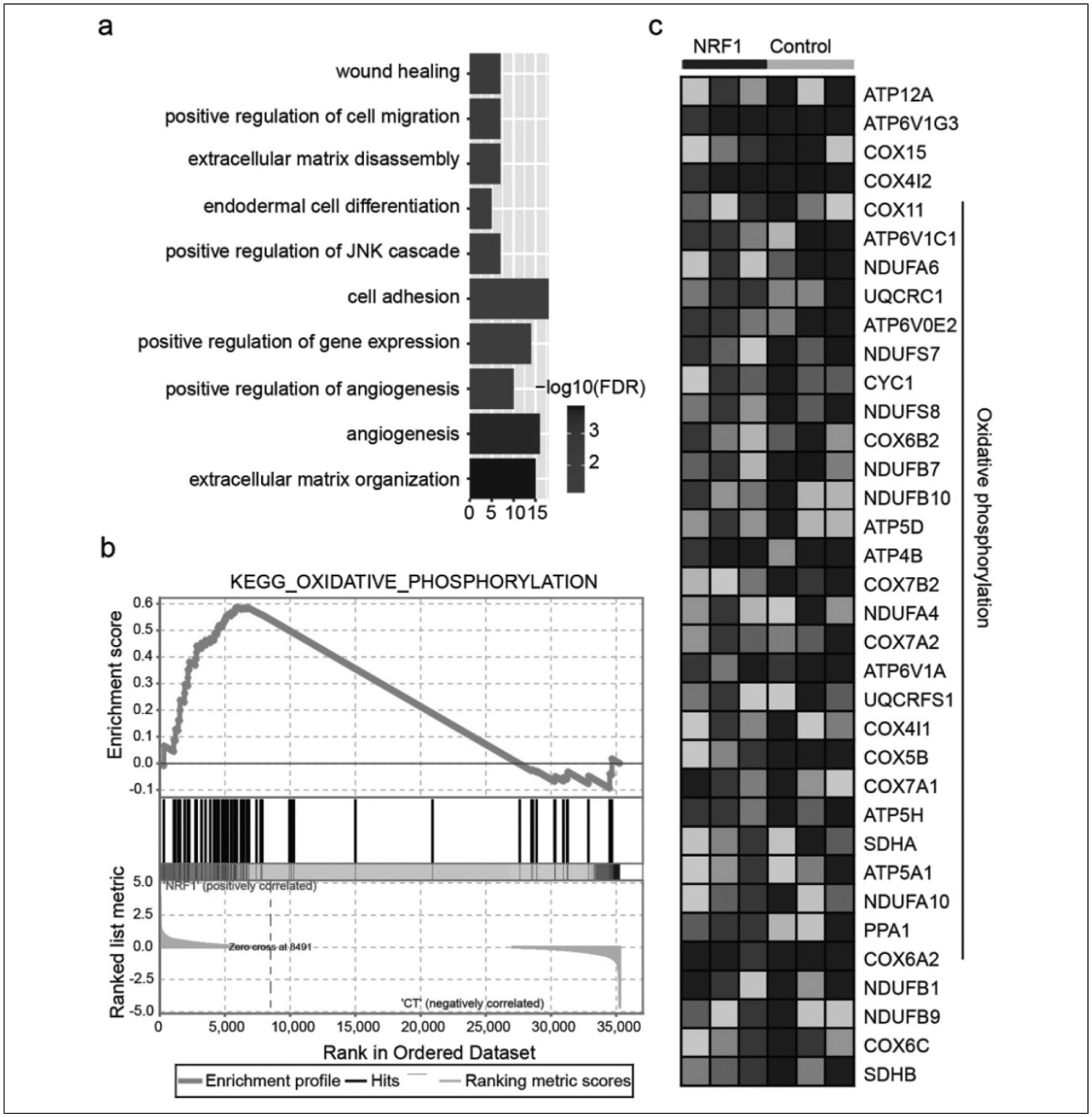

Figure 2. NRF1 is significantly associated with biological processes related to tumor metastasis and promotes the expression of OXPHOS genes. (a) GO functional annotation for upregulated genes in M10A-NRF1-OE and vOE cells. Top 10 of GO enrichments were showed, n = 3. (b) GSEA analysis of differentially expressed genes (DEGs) in M10A-NRF1-OE and vOE cells, n = 3. (c) Heatmap of OXPHOS genes in M10A-NRF1-OE and vOE cells, n = 3.

Abbreviations: NRF1, nuclear respiratory factor 1; OXPHOS, oxidative phosphorylation; GSEA, Gene Set Enrichment Analysis.

(Beyotime Institute of Biotechnology, Shanghai, China). Mitochondrial DNA (mtDNA) content was measured by real-time PCR analysis and determined by assessing the relative levels of human cytochrome oxidase 1 (H-mtCO1) versus H-β-globin.

ATP Assay

ATP levels were determined using an enzymatic coupled assay as previously described.[11] In brief, ATP levels in suspension versus adhesion cells were determined using an ATP assay kit (Sigma-Aldrich, St Louis, MO, USA) and according to the manufacturer's protocol. For one reaction, 2.5×10^4 suspension or adhesion cells were used. The ATP standard with a solution of 1 μM was used as an internal control. After the reaction, the bioluminescence was examined by VICTOR X4 Multilabel Plate Reader (PerkinElmer). The ATP levels were determined by luminescence intensity normalized by protein content of each reaction examined using BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). All the measurements were replicated three times.

RNA Extraction and Quantification

Total RNAs were extracted from cells using the RNAeasy Mini Kit (QIAGEN) and reverse transcribed using the ABI reverse transcription kit according to the manufacturer's instructions. The reaction of real-time PCR was performed in Power SYBR Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA, USA) on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The relative expression levels were expressed in arbitrary units where the C_t value of the gene of interest was normalized to that of RPL19. The primers for qRT-PCR were designed using the Primer Express software (Version 2.0, Applied Biosystems). The primers used in real-time PCR were as follows : NRF1 Forward Primer 5'-AGTGCTTA GCCCTTGATGAAGA-3'and Reverse Primer 5'-GCTCTG AAGTGACCTCTGGTAT-3'; GPX1Forward Primer 5'-CAGT CGGTGTATGCCTTCTCG-3' and Reverse Primer 5'-GAG GGACGCCACATTCTCG-3'; SOD2 Forward Primer 5'-GCT CCGGTTTTGGGGTATCTG-3' and Reverse Primer 5'-GCGTTGATGTGAGGTTCCAG-3'.

Measurement of Mitochondrial Membrane Potential ($\Delta\Psi M$)

ΔΨM was detected in cells by JC-1 detection kit (Biyuntian (C2006)) according to the instructions of the kit, 6×10^5 cells were collected, and were resuspended in 0.5 ml of cell culture medium, then 0.5 ml of JC-1 staining working solution were added. We incubated it at 37°C for 20

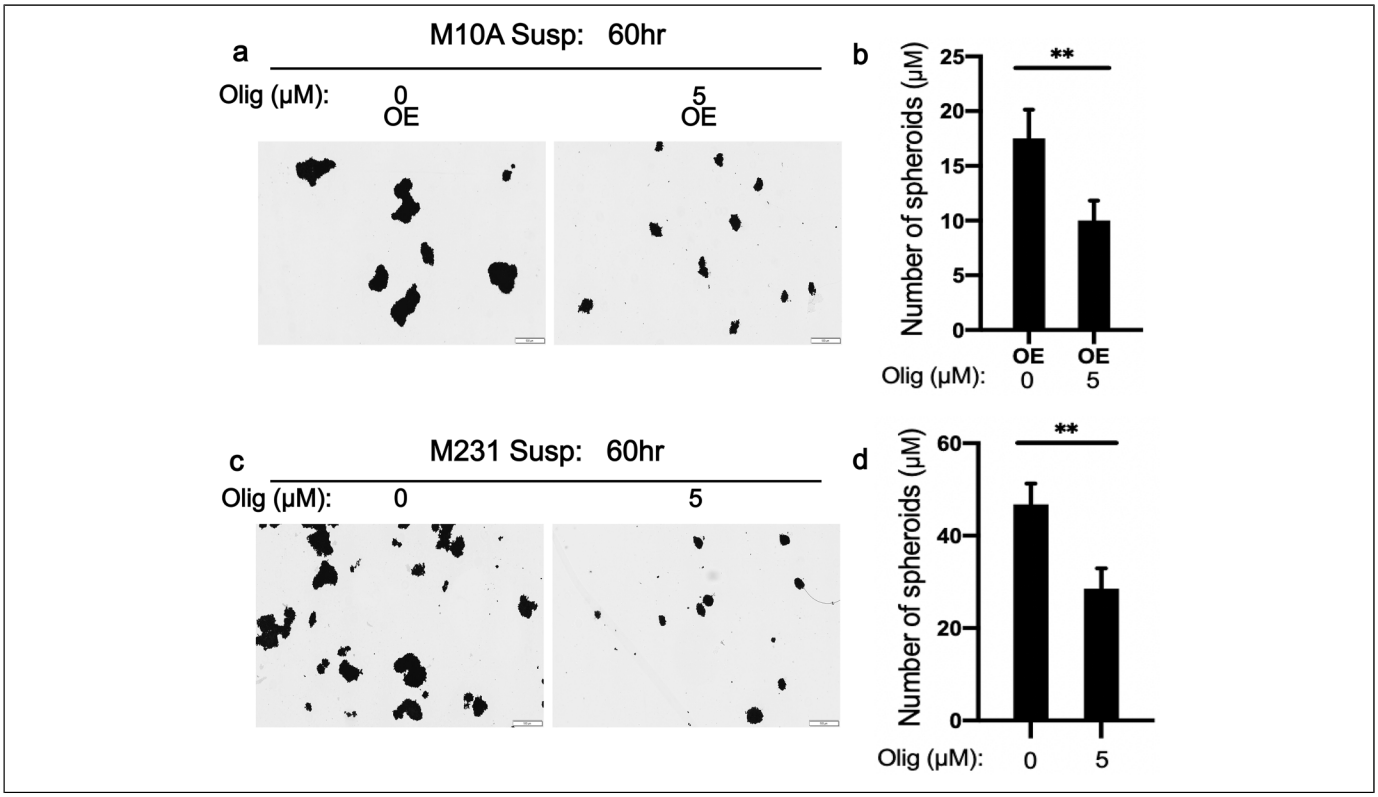

Figure 3. NRF1 inhibits anoikis through the oxidative phosphorylation pathway. (a-b): The number and size of spheres were analyzed in oligomycin A treated breast cancer cells (M10A). Three view fields were selected to count the number of cells and data was presented as mean ± SD, n = 3. (c-d) The number and size of spheres were analyzed in oligomycin A treated breast cancer cells (M231).Three view fields were selected to count the number of cells and data was presented as mean ± SD, n = 3. *P < .05, **P < .01, and ***P < .001.
Abbreviations: NRF1, nuclear respiratory factor 1.

minutes. After incubation, we discarded the supernatant and washed it twice with JC-1 staining buffer (1×), centrifuge at 600g for 3 to 4 minutes at 4°C, and pellet the cells. After the centrifugation, we discarded the supernatant and used an appropriate amount of JC-1 staining buffer (1×). After resuspension, we performed a flow cytometry analysis.

Western Blot

Western blot analysis was performed as previously described.[15] Briefly, after collecting protein lysates in 2% SDS, we measured the protein concentration of each sample. Next, the proteins were transferred to a membrane, the membrane was blocked and then treated with NRF1(Cell Signaling Technology, 69432S) (1:1000), ACTB (beta-actin) (Cell Signaling Technology, 4970T) (1:1000), N-Cadherin (CDH2) (Cell Signaling Technology, 4061S) (1:1000), E-Cadhein (CDH1) (Cell Signaling Technology, 3195T) (1:1000), Vinentin (VIM) (Cell Signaling Technology, 5741T) (1:1000), Snail (SNAI1) (Cell Signaling Technology, 3879T) (1:1000) and ZO-1 (TJP1) (Cell Signaling Technology, 13663S) (1:1000) antibodies (with washing between steps), and target protein bands were detected by an enhanced chemiluminescence system (GE Healthcare Life Sciences, Chalfont, UK).

Statistical Analysis

RNA was extracted from M10A-NRF1-OE and vOE cells using RNAiso reagent from TaKaRa (Dalian, China). The RNA sequencing was performed by Novogene Corporation (Beijing, China). The analysis for differential expression analysis was done by the use of DESeq2 R package. Gene Set Enrichment Analysis (GSEA) was conducted using the software downloaded from the GSEA website (http://software.broadinstitute.org). Statistical analyses were performed using GraphPad Prism 8 software and statistical significance was defined as P value less than .05. All results are shown as mean ± SEM (standard error of the mean).

Results

NRF1 Promotes Mitochondrial Biosynthesis, ATP Generation, and Enhances Mitochondrial Membrane Potential

Previous studies have shown that upregulated NRF1 expression promotes the malignant transformation of nontumorigenic MCF10A (M10A), while NRF1 knockdown in MDA-MB-231 (M231) cells alters tumor cell invasion and metastasis.[11] Metabolic reprogramming of cancer cells affects malignant

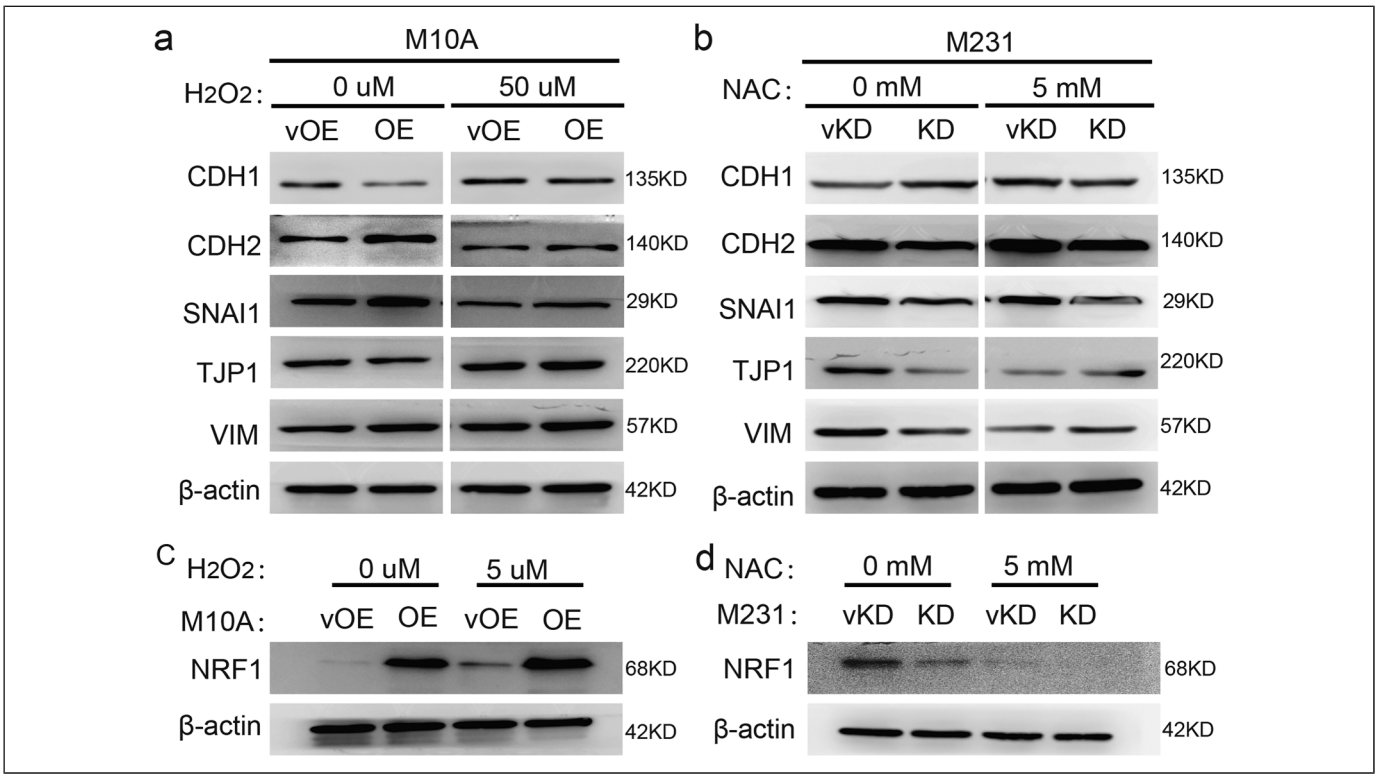

Figure 4. NRF1 can affect EMT by regulating ROS homeostasis. (a) The expression of CDH1, CDH2, SNA1, VIM and β-actin were detected in H_2O_2 50 (umol/L) treated M10A-NRF1-OE cells by Western blot. (b) The expression of CDH1, CDH2, SNA1, VIM, and β-actin were detected in NAC 5 (mmol/L) treated M231-NRF1-OE by Western blot. (c) The expression levels of NRF1 were detected in H_2O_2 50 (umol/L) treated M10A-NRF1-OE cells by Western blot. (d) The expression levels of NRF1 in NAC 5 (mmol/L) treated M231-NRF1-OE by Western blot.Abbreviations: NRF1, nuclear respiratory factor 1; EMT, epithelial mesenchymal transition.

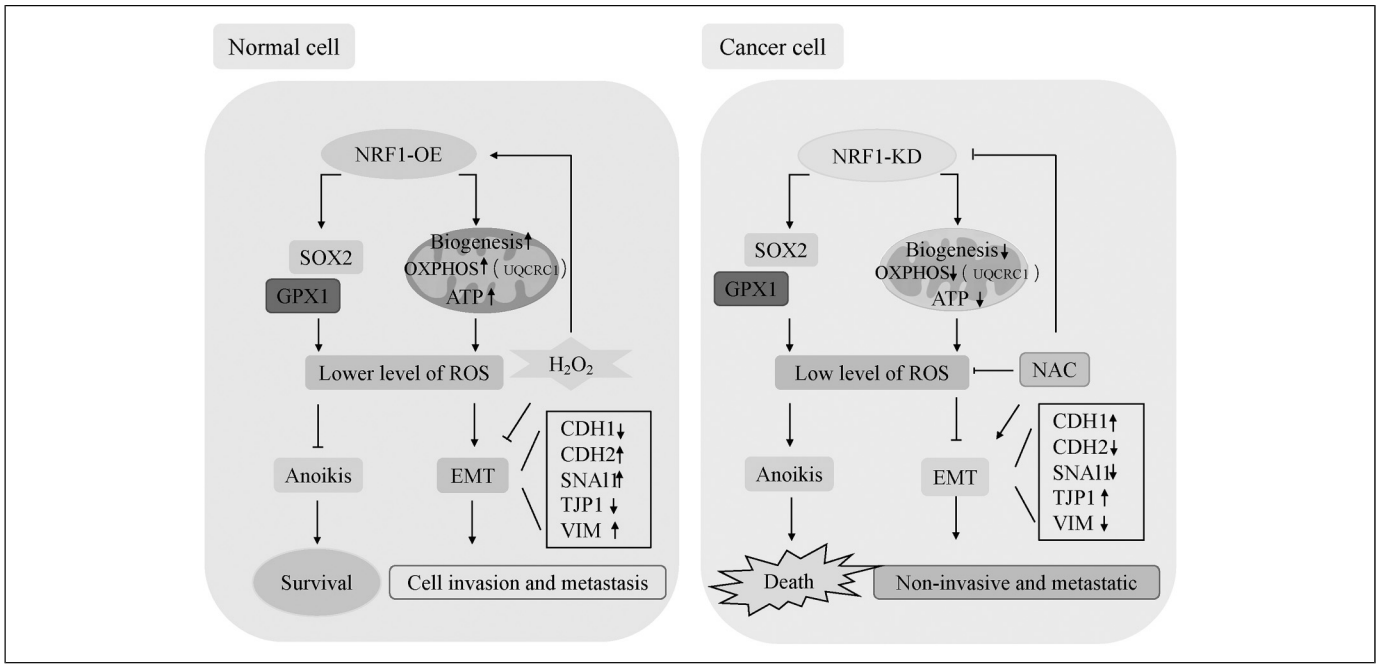

Figure 5. A diagram of NRF1 modulation of ROS homeostasis in breast cancer. The figure shows that NRF1 enhances EMT and anoikis resistance in breast cancer by regulating ROS homeostasis. In normal mammary epithelial cells, NRF1 overexpression increases ROS level and ATP production by activating the OXPHOS pathway, and consequently promotes EMT and anoikis resistance, furthermore NRF1 overexpression promotes the expression of ROS scavenging enzymes SOX2 and GPX1. Additionally, exogenous ROS can stimulates the expression of NRF1 and ROS scavenger enzymes to reduce ROS levels and thus inhibit EMT. NRF1 knockdown impairs the redox homeostasis in tumor cells by interrupting OXPHOS pathway and decrease the expression of ROS scavenging enzymes, thus promote MET.
Abbreviations: NRF1, nuclear respiratory factor 1; EMT, epithelial mesenchymal transition; OXPHOS, oxidative phosphorylation.

transformation and tumor development, including invasion and metastasis.[16] To investigate the effect of overexpression or knockdown of NRF1 in mitochondrial function in breast cancer cells. We constructed NRF1-overexpressed M10A and NRF1-knockdown M231 cell lines (Figure S1a-d). We found that upregulated NRF1 expression significantly promoted ATP generation and mitochondrial biogenesis in both adherent and detached M10A cells (Figure 1a and 1c). In addition, upregulated NRF1 showed a higher mitochondrial membrane potential, which led to more ATP and ROS generation (Figure 1e and 1f). Similarly, NRF1 knockdown in breast cancer cell M231 reduced ATP generation (Figure 1b), mitochondrial biosynthesis (Figure 1d) and attenuated ROS generation (Figure 1g and 1h).

The above results indicate that NRF1 is an important regulator involved in mitochondrial biogenesis and energy metabolism, promoting ROS generation, ATP generation, and enhancing mitochondrial membrane potential in breast cancer.

NRF1 Expression is Associated With Genes Responsible for Mitochondrial Metabolism

To find the key signaling pathways induced by NRF1 in breast cancer. Transcriptome sequencing was performed on M10A-NRF1-OE and control cells. We found that the upregulated genes in M10A-NRF1-OE cells were enriched in multiple biological processes, such as angiogenesis, cell migration, and extracellular matrix reorganization (Figure 2a). The GSEA showed that the activation of OXPHOS was related to NRF1 overexpression (FDR < 0.25) (Figure 2b). As shown in Figure 2c, OXPHOS genes were significantly upregulated in NRF1-OE cells, containing the electron transport chain, ATP synthase, cytochrome C subunit, and NADH dehydrogenase genes. These results suggest that upregulated NRF1 expression can promote the expression of OXPHOS genes and enhance the level of OXPHOS in breast cancer cells.

NRF1 Inhibits Anoikis Through the Oxidative Phosphorylation Pathway

Anoikis is a barrier to cancer metastasis.[17] Our results revealed that NRF1 overexpression promotes the malignant transformation of nontumorigenic M10A and promotes the expression of OXPHOS genes. To determine the relationship between OXPHOS and anoikis. We treated M10A-NRF1-OE cells and M231 parental cells with oligomycin A, and set up a control group. After 60 hours of detached culture, we found that oligomycin A reduced the number and size of M10A-NRF1-OE spheroids (Figure 3a and 3b). Likewise, both size and number of spheroids of M231 cells decreased after treated with oligomycin A (Figure 3c and 3d). Our results show that oligomycin A inhibits the process of OXPHOS in NRF1 overexpressed cells. These results provide strong evidence that NRF1 inhibits anoikis through the OXPHOS pathway.

ROS Homeostasis Regulated by NRF1 Is Dispensable for EMT

Cancer cells may acquire migratory and invasive phenotypes by reducing anoikis.[18] We have shown that NRF1 can inhibit anoikis through the OXPHOS pathway. The mitochondria-associated genes are upregulated in response to NRF1 overexpression. Most intracellular ROS are by-products of mitochondrial OXPHOS. Low levels of ROS promote tumor development by promoting tumor growth, invasion, and metastasis, whereas excessive cellular levels of ROS result in cell death through irreversible damage to proteins, nucleic acids, and lipids.[19,20] To validate the relationship between NRF1, ROS homeostasis, and EMT in breast cancer cells. We treated M10A and M231 cells with H_2O_2 and the ROS inhibitor N-acetylcysteine (NAC), respectively. Our western blot result reveals that untreated M10A showed that upregulated NRF1 expression significantly promoted the expression of mesenchymal markers, including CDH2, SNA1, and Vimentin. H_2O_2 reverses increased EMT induced by NRF1 overexpression in M10A cells (Figure 4a). Interestingly, our finding shows that ROS were maintained at low levels in M10A-NRF1-OE cells, and we found that NRF1 promoted the expression of ROS scavenging enzymes GPX1 and SOD2 (Figure S1e). Alternatively, untreated M231-NRF1-KD cells indicate the downregulation of EMT markers, while the NAC treatment M231-NRF1-KD indicated the upregulation of EMT markers, and NAC rescues the decreasing EMT induced by knockdown of NRF1 in M231 cells (Figure 4b). It has been shown that NRF1 expression contributes to the maintenance of normal redox homeostasis.[21] Moreover, ROS levels play an important role in tumor proliferation and metastasis.[22,23] Exploring the relationship between ROS and NRF1 will help better understand the mechanism of tumor proliferation and metastasis. Therefore, we treated M231-NRF1-KD and M10A-NRF1-OE cells with H_2O_2 and NAC and then detected the expression of NRF1. We found that H_2O_2 stimulation upregulates the expression of NRF1 in M10A cells (Figure 4c). Upregulated NRF1 expression induced more ROS scavenging enzymes GPX1 and SOD2, which reduce ROS levels and maintain normal homeostasis. Alternatively, NAC treatment decreases the expression level of NRF1 in M231 cells (Figure 4d). As a result, NRF1 stimulates EMT and regulates ROS homeostasis, resulting in increased tumor proliferation and metastasis.

Discussion

Tumor metastasis is one of the most life-threatening pathological events associated with malignant tumors.[24] Renewed attention has been focused on altered metabolic programs that underpin the metastatic process in breast cancer.[25] Understanding the molecular mechanisms of metabolic underlying metastatic breast cancer cells may reveal potential vulnerabilities suitable for therapeutic targeting.

Our study demonstrated that NRF1 overexpression enhances mitochondrial membrane potential, mitochondrial biosynthesis, and ATP production in breast cancer cells. We revealed that NRF1 elevated genes involved in mitochondrial OXPHOS by transcriptome sequencing of the M10A-NRF1-OE cell line.

NRF1 is a multifunctional protein highly expressed in human breast cancer tissues and is involved in various biological processes, including mitochondrial metabolism, cell cycle, chromatin structure, apoptosis, cell adhesion/invasion, and DNA repair.[26–28] Hence, combining the present and previous findings,[11] we speculate that NRF1 might play a putative role in regulating mitochondrial energy metabolism in breast cancer. Therefore, exploring the relationship between NRF1 and cancer metastasis in breast cancer has significance for treating breast cancer.

Furthermore, we inhibit the OXPHOS pathway with oligomycin A, and we found that NRF1 enhances anoikis resistance. This suggests that NRF1 enhances the OXPHOS pathway to suppress anoikis and promote tumor proliferation. The enhanced mitochondrial OXPHOS pathway can generate a large amount of ATP to meet the energy required for tumor proliferation and metastasis, and sufficient energy is necessary for anoikis resistance and regulation of EMT phenotype in breast cancer cells.[29]

ROS generated as a byproduct of OXPHOS plays an important role as signalling molecules in physiological and pathological process.[30,31] Radisky et al and Rhyu et al studies found that ROS is a crucial factor in promoting EMT in certain cell types.[32–34]EMT promotes tumor metastasis, enables cancer cells to evade an unfavorable environment, and increases anoikis resistance.[35] Furthermore, the study found that ROS promotes EMT at low levels.[36] However, in this study, NRF1 overexpression promotes EMT in breast cancer cells. We found that NRF1 overexpression can regulate the expression of ROS scavenging enzymes and maintain low levels of intracellular ROS. This may explain why NRF1 overexpression promotes EMT.

NFF1 is essential for maintaining normal redox homeostasis in the Human HepG2 cells.[37] In our study, we demonstrated that exogenous ROS levels could regulate the expression of NRF1 when breast cancer cells are exposed to oxidative stress. NRF1 maintains intracellular ROS levels through the expression of ROS scavenging enzymes. Our findings highlighted the critical role of NRF1 in maintaining intracellular ROS homeostasis (Figure 5).

In recent years, therapeutic strategies have been aimed at disrupting redox homeostasis in cancer cells, which is a potential field for developing new anticancer drugs. NRF1 regulates metabolic reprogramming, maintains intracellular ROS homeostasis, and promotes tumor proliferation and metastasis in breast cancer, which can adversely affect or undermine cancer treatment outcomes. A potential target for breast cancer treatment is to influence NRF1 expression, which can influence tumor proliferation and migration.

Based on our findings, combined therapies targeting gene inhibitors and ROS inducers have the potential to offer promise for tumor therapy. However, the correlation between NRF1 and ROS was not explored due to the difficulty of measuring ROS levels in vivo.

Conclusion

Our findings suggest that NRF1 is a potential therapeutic target for preventing or relieving breast cancer, and novel therapeutic drugs can be developed against NRF1 to perturb the metabolism of cancer cells for cancer treatment.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Statement

Our study did not require an ethical board approval because it did not contain human or animal trials.

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Supplemental Material

Supplemental material for this article is available online.

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