

PINK1-mediated mitophagy enhances breast cancer proliferation through metabolic reprogramming

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Abstract. Breast cancer is a predominant cause of cancer-related mortality among women, particularly aggressive subtypes such as triple-negative breast cancer (TNBC), which currently lack effective targeted therapies. While PTEN-induced kinase 1 (PINK1) is known for its role in maintaining mitochondrial homeostasis via mitophagy, its specific contributions to breast cancer progression and metabolic regulation remain poorly defined. The present study aimed to investigate the oncogenic potential of PINK1 and its influence on metabolic reprogramming. To achieve this, the PINK1 expression levels in breast cancer tissues and cell lines were assessed. Gain- and loss-of-function methodologies were employed in luminal (MCF-7) and TNBC (MDA-MB-231) cells. Then, mitophagy was evaluated by measuring LC3-II levels, Parkin expression and utilizing transmission electron microscopy. Glucose uptake assays and metabolite quantification (including pyruvate and acetyl-CoA) were conducted. Reverse transcription-quantitative polymerase chain reaction identified phosphoglycerate kinase 2 (PGK2) as a downstream target of PINK1. Functional assays were then performed to

examine the proliferation, migration and invasion of cells with PINK1 overexpression. The results demonstrated that PINK1 overexpression increased mitophagy and induced a glycolytic phenotype, characterized by enhanced glucose uptake and elevated PGK2 levels. Elevated concentrations of pyruvate and acetyl-CoA indicated increased metabolic flux. Functionally, PINK1 promoted proliferation, migration and invasion in both cell types. Knockdown of PGK2 reversed these effects, underscoring its critical role in PINK1-mediated metabolic reprogramming. Transcriptomic data obtained from online databases revealed a correlation between high PINK1 expression and immunosuppressive tumor microenvironments, as well as poor prognosis. The PINK1-PGK2 axis constitutes a critical mechanism linking mitophagy to glycolytic reprogramming in breast cancer, representing a novel therapeutic target, particularly for TNBC. Targeting this axis may yield new strategies for addressing treatment-resistant, metabolically adaptive breast cancer.

Introduction

Breast cancer is the most prevalent cancer among women globally. GLOBOCAN estimated there would be 2.3 million new cases of breast cancer in 2022; breast cancer also caused 670,000 deaths in 2022, accounting for 25.0% of female cancer cases and 15.5% of cancer-related deaths (1). Among its subtypes, triple-negative breast cancer (TNBC) is particularly aggressive as it lacks hormone receptors and HER2 expression. This limitation restricts the availability of targeted therapies for patients with TNBC (2-4). The aggressive nature of TNBC necessitates the urgent elucidation of novel molecular drivers of its progression.

Emerging evidence indicates that metabolic reprogramming constitutes a fundamental hallmark of tumor progression. This characteristic is especially critical in rapidly proliferating tumors such as TNBC, which exhibit heightened demands for energy and biosynthetic resources (5-7). The Warburg effect (8), characterized by aerobic glycolysis, is well-documented. However, mitochondrial function, including

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oxidative phosphorylation and dynamics, plays a critical role in facilitating tumor cell survival, proliferation and adaptation to stress (9,10). Mitochondria are essential components of cellular metabolism and the preservation of their integrity is imperative (11).

Mitophagy, the selective degradation of damaged mitochondria, is crucial for maintaining mitochondrial quality and regulating cellular metabolism (12-14). PTEN-induced kinase 1 (PINK1) plays a central role in this process (15). Under normal conditions, PINK1 is rapidly degraded; however, upon mitochondrial damage, it accumulates on the outer membrane and recruits the E3 ligase Parkin to initiate mitophagy (16). While PINK1 has been recognized for its neuroprotective role in Parkinson's disease, studies have also revealed its dualistic function in cancer biology (17-19). In breast cancer, particularly TNBC, high PINK1 expression is associated with poor prognosis (20). Notably, PINK1 exhibits context-dependent roles in cancer, suppressing tumorigenesis in liver and renal cancer while correlating with poor prognosis in breast, ovarian and lung cancer (21-24).

Mechanistically, PINK1-mediated mitophagy is hypothesized to facilitate cancer progression; it achieves this by maintaining mitochondrial integrity, ensuring ATP production and alleviating oxidative stress (25,26). A recent investigation indicated that PINK1 may also modulate glycolytic flux by regulating key glycolytic enzymes (20). PINK1-driven mitophagy interacts with metabolic reprogramming by upregulating phosphoglycerate kinase 2 (PGK2) to enhance glycolytic flux, increasing glucose uptake and elevating pyruvate/acetyl-CoA levels; this interplay promotes TNBC pathogenesis. Specifically, this mechanism could be crucial for driving tumor growth, facilitating migration and developing resistance to therapies in TNBC (27). PGK2 is a notable enzyme in the glycolytic pathway, catalyzing the conversion of glycerol-1,3-diphosphate into 3-phosphoglycerate, while simultaneously producing ATP (28). Furthermore, PGK2 influences DNA replication and repair in mammalian nuclei (29,30). PGK2 expression is regulated by oxygen tension, with elevated expression levels often correlating with accelerated tumor growth and a stronger anaerobic growth phenotype (31). Emerging evidence suggests that PGK2, traditionally regarded as testis-specific, is also expressed in various malignancies (32). PGK2 is a specific form found in spermatogenic cells and in renal, breast, pancreatic, ovarian and testicular cancer cells (33,34). Studies have emphasized the role of PGK2 in cancer, particularly its upregulation in ovarian cancer, linking it to metabolic reprogramming and tumor progression (35,36). However, its precise role in breast cancer remains to be elucidated.

The present study aimed to investigate the potential oncogenic roles of PINK1 in breast cancer, emphasizing its involvement in metabolic reprogramming through mitophagy. Specifically, the present study explored whether PGK2 acts as a downstream effector of PINK1-associated metabolic regulation. Using gain- and loss-of-function approaches, the present study examined the relationship between PINK1-mediated mitophagy and glycolytic metabolism, as well as its potential impact on malignant phenotypes in luminal and TNBC cells. By elucidating the link between mitochondrial quality control and metabolic reprogramming, this work sought to provide

insights into the role of the PINK1-PGK2 axis in breast cancer progression and its potential therapeutic relevance, particularly in TNBC.

Materials and methods

Data collection. Gene expression data for patients with breast cancer were downloaded from the Xena database (<https://xenabrowser.net/datapages/>). The dataset includes 1,226 samples, consisting of 1,113 breast cancer tumor samples and 113 normal control samples, which were collected using Illumina HiSeq 2000 sequencing technology (37). These data correspond to The Cancer Genome Atlas-Breast Invasive Carcinoma (TCGA-BRCA) project (dbGaP: phs000178). The original study describing this dataset was conducted by TCGA Network (<https://portal.gdc.cancer.gov/>) (38). This dataset was used to explore the gene expression profiles of patients with breast cancer, providing insight into the molecular mechanisms involved in tumor progression and the role of PINK1-mediated mitophagy.

Screening of differentially expressed genes (DEGs). To identify DEGs associated with mitochondrial autophagy in breast cancer, transcriptomic data from the TCGA-BRCA dataset were utilized. Tumor and normal control samples were categorized into the Tumor and Normal groups. Differential gene expression analysis was conducted using the DESeq2 package in R (v4.3.1) (<https://www.r-project.org/>), with a threshold for selecting DEGs set at false discovery rate (FDR) <0.05 and log2 fold change (FC) >1. Significant DEGs were visualized using heatmaps generated with the heatmap package and volcano plots created with ggplot2, both implemented in the R statistical environment. Subsequently, the selected DEGs underwent functional enrichment analysis utilizing the clusterProfiler package in R (v4.3.1). Gene Ontology (GO) (<http://geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp/>) (39) pathway enrichment analyses were performed to identify the biological processes and signaling pathways associated with the DEGs. An FDR threshold of <0.05 was used to define significant enrichments.

Differential expression of mitophagy and glycolysis genes. To identify DEGs associated with mitophagy and glycolysis in breast cancer, mitochondrial autophagy-related gene sets were compiled from the literature and the MSigDB database (40). The mitophagy gene sets were obtained and curated using Metascape (<https://metascape.org/>), including GOBP_MITOPHAGY, GOBP_PARKIN_MEDIATED_STIMULATION_OF_MITOPHAGY_IN_RESPONSE_TO_MITOCHONDRIAL_DEPOLARIZATION, GOBP_POSITIVE_REGULATION_OF_MITOPHAGY, GOBP_POSITIVE_REGULATION_OF_MITOPHAGY_IN_RESPONSE_TO_MITOCHONDRIAL_DEPOLARIZATION, GOBP_REGULATION_OF_MITOPHAGY, REACTOME_MITOPHAGY, REACTOME_PINK1_PRKN_MEDIATED_MITOPHAGY and REACTOME_RECEPTOR_MEDIATED_MITOPHAGY. Similarly, glycolysis-related gene sets, such as BIOCARTA_GLYCOLYSIS_PATHWAY, GOBP_GLYCOLYTIC_PROCESS_THROUGH_FRUCTOSE_6_

PHOSPHATE, GOBP_GLYCOLYTIC_PROCESS_THROUGH_GLUCOSE_6_PHOSPHATE, HALLMARK_GLYCOLYSIS, KEGG_GLYCOLYSIS_GLUONEOGENESIS, KEGG_MEDICUS_REFERENCE_GLYCOLYSIS, MOOTHA_GLYCOLYSIS, REACTOME_GLYCOLYSIS and WP_GLYCOLYSIS_AND_GLUONEOGENESIS, were also downloaded from the same databases. Next, the genes listed under each category were extracted, duplicates were removed and they were categorized into two groups: Mitophagy-related genes and glycolysis-related genes. The intersection of the DEGs with these gene sets were then identified. The overlapping genes for mitophagy and glycolysis were termed Mito-DEGs and Glyco-DEGs, respectively, and were used for subsequent analysis.

Enrichment and correlation analysis of mitophagy and glycolysis. Utilizing the expression matrix from the TCGA-BRCA cohort, single sample gene set enrichment analysis (ssGSEA) was performed to calculate enrichment scores for the mitophagy and glycolysis gene sets in each sample (41). To assess the statistical differences between the Tumor and Normal groups, the Wilcoxon rank-sum test was applied (42). A significance threshold of $P < 0.05$ $\log_2 FCI > 1$ was established. Results were visualized using ggplot2 to illustrate the differential enrichment of pathways between the two groups. Furthermore, based on the differential gene expression results from the Tumor and Normal groups, the GSEABase package in R (v4.3.1) was utilized to analyze the enrichment of the mitophagy and glycolysis gene sets (43). The enrichplot package in R was subsequently employed to generate enrichment result plots. To evaluate the correlation between mitophagy and glycolysis, the Spearman correlation coefficient was calculated based on the enrichment scores of the respective gene sets in the tumor samples. Statistical significance for these correlations was defined as $P < 0.05$. The relationship between differential glycolysis genes and mitophagy pathway enrichment scores was also analyzed using Spearman's correlation coefficient. The results of these correlation analyses were visualized using scatter plots.

PINK1 single-gene functional enrichment analysis. Based on the expression levels of PINK1 in the Tumor samples from the TCGA-BRCA cohort, the samples were divided into two groups: PINK1-high and PINK1-low, using the median expression value as the threshold. DEGs between these two groups were identified using the DESeq2 package in R (44). A threshold of $FDR < 0.05$ and $\log_2 FCI > 1$ was applied to select significant DEGs. The DEGs were visualized using heatmaps created with the heatmap package and volcano plots generated with ggplot2 (45). Subsequently, functional enrichment analysis of the selected DEGs was performed using the clusterProfiler package in R (46). GO functional annotation and KEGG pathway enrichment analysis were conducted, with an FDR threshold of < 0.05 to define significant enrichment (46).

Immune-related analysis. The CIBERSORT algorithm (47) (<https://cibersort.stanford.edu/index.ph>) was utilized to calculate the infiltration scores of 22 immune cell types in the breast cancer samples. The correlation between PINK1 expression levels and the distribution of these immune cell proportions

was then analyzed using the Spearman correlation coefficient test in R (v4.3.1) (48). A threshold of $P < 0.05$ was set to define significant correlations. Next, cancer-associated fibroblasts (CAFs), M1 macrophage and M2 macrophage-related genes were collected from the literature. The Spearman correlation coefficient was calculated to assess the relationship between PINK1 expression and the expression of these genes. $P < 0.05$ was used to determine significant correlations. The correlation analysis results were visualized using scatter plots, and the top three genes with the highest correlation coefficients for each group were displayed.

Prognostic correlation analysis. To evaluate the prognostic significance of PINK1 and the related gene sets, gene expression data were integrated with clinical survival outcomes. This was achieved by combining individual gene expression levels with ssGSEA enrichment scores for specific gene sets. These gene sets were selected based on their relevance to processes such as mitophagy, metabolism and the immune response. The survival analysis was conducted using the survival package (v2.41-1) in R (v4.3.1) (49). Kaplan-Meier survival curves and log-rank tests were used to compare survival outcomes between high and low expression groups for each gene or gene set. Additionally, Cox proportional hazards regression models were employed to assess the independent prognostic value of PINK1 and its associated genes. This comprehensive analysis aimed to identify potential biomarkers and to improve the understanding of how PINK1-related pathways influence breast cancer prognosis, providing insights for patient stratification and targeted therapeutic approaches.

Cell lines and cell culture. Two breast cancer cell lines, MCF-7 (hormone receptor-positive) and MDA-MB-231 (triple-negative and highly invasive) were procured from the Cell Bank, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Chinese Academy of Sciences. MCF-7 cells, recognized for their hormone-dependent characteristics, and MDA-MB-231 cells, noted for their aggressive metastatic potential, were selected to represent distinct molecular subtypes of breast cancer (50). MCF-7 cells were cultured in RPMI-1640 medium (Procell Life Science & Technology Co., Ltd.; cat. no. PM150110), while MDA-MB-231 cells were maintained in L-15 medium (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 11415064) at 37°C with 5% CO_2 . All cell lines underwent short tandem repeat profiling and mycoplasma testing to verify authenticity and sterility. All cell lines were cultured in media supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Passage numbers were kept below 20 for consistency. Routine mycoplasma testing was performed using the MycoAlert Mycoplasma Detection Kit (Lonza Group Ltd.; cat. no. LT07-218) to ensure cell culture integrity.

Cell transfection. To evaluate the functional impact of PINK1, four experimental groups were established: Empty vector negative control (OE-NC), PINK1 overexpression (PINK1-OE), short hairpin RNA (shRNA) targeting PINK1 (sh-PINK1) and sh-NC. Constructs for PINK1-OE and sh-PINK1 were generated using pLV3 lentiviral vector (MiaoLing Bio; lot. no. P693631) incorporating *NotI/XhoI* restriction sites for the

insertion of the human PINK1 coding sequence (NCBI accession NM_032409.2). OE-NC and sh-NC comprised empty plasmids. PINK1 overexpression plasmids (lot. no. P69631) and sh-PINK1 constructs (lot. no. P50879) were obtained from Wuhan MiaoLing Biotech Science Co., Ltd. Constructs for PINK1-OE and silencing PGK2 (siPGK2) were used to assess the effects of metabolic suppression in breast cancer cells. Transfection was conducted using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.; cat. no. L3000015) following the manufacturer's protocol. Specifically, 2 μ g of plasmid DNA was mixed with 100 μ l of Opti-MEM I medium (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 31985070), and 6 μ l of Lipofectamine 3000 was separately diluted in another 100 μ l of Opti-MEM I. The two solutions were combined, incubated for 15 min at room temperature and then added dropwise to MCF-7 and MDA-MB-231 breast cancer cells seeded in 6-well plates. Cells were incubated for 24 h post-transfection prior to downstream assays at 37°C. This methodology ensured efficient modulation of PINK1 expression for assessing its role in mitophagy and metabolic remodeling in breast cancer cells. For siRNA transfection, two siRNA oligonucleotides targeting PGK2 were designed: siRNA-1 sense, 5'-GAAGUUGACUUUAGACAAA-3' and antisense, 5'-UUUGUCUAAAGUCAACUUC-3'; siRNA-2 sense, 5'-GGGACAAGUUUGACGAGA-3' and antisense, 5'-UUCUCGUCAAACUUGUCCC-3'. In the experiment, an NC siRNA (siNC), sense 5'-UUGAUGUGUUUAGUCGCUAtt-3' and antisense 5'-UAGCGACUAAACAUCAAAtt-3' was included to rule out non-specific effects. For PINK1 knockdown, a shRNA targeting the sequence 5'-GAAGCCACCATGCCTACATTGT-3' was used. sh-NC targeted the sequence 5'-GCGTGATCTTCACCGACAAGA-3'.

Chemical synthesis of siPGK2. PGK2-targeting siRNA sequences were designed from human PGK2 mRNA (NCBI RefSeq: NM_138733.5). RNA oligonucleotides were synthesized by solid-phase phosphoramidite chemistry on a CPG column (Glen Research; cat. no. 20-3330; 1000 Å, 1 μ mol) using 2'-O-TBDMS RNA phosphoramidites (Glen Research; Bz-A-CE, cat. no. 10-3003; Ac-C-CE, cat. no. 10-3015; Ac-G-CE, cat. no. 10-3025; U-CE, cat. no. 10-3030) on an Applied Biosystems 394 DNA/RNA Synthesizer (Thermo Fisher Scientific, Inc.). Oligonucleotides were cleaved and base-deprotected with a ammonium hydroxide-methylamine mixture [1:1 ammonium hydroxide, Sigma-Aldrich (Merck KGaA), cat. no. A2706; methylamine, Sigma-Aldrich (Merck KGaA), cat. no. M2769], followed by 2'-O-TBDMS removal using triethylamine trihydrofluoride (Sigma-Aldrich; Merck KGaA; cat. no. 344648) in DMSO (65°C, 2.5 h). RNAs were purified by RP-HPLC (Agilent 1260 Infinity; ZORBAX Eclipse Plus C18; 4.6x250 mm, 5 μ m; cat. no. 959963-902) and annealed to generate duplex siRNA.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays. For RT-qPCR, total RNA was extracted from MCF-7 and MDA-MB-231 breast cancer cells across the OE-NC, PINK1-OE, sh-NC, sh-PINK1, si-NC and si-PGK2 experimental groups. RNA isolation was carried out using TRIzol reagent (Beyotime Biotechnology; cat. no. R1100) in accordance with the manufacturer's instructions. First-strand

cDNA was synthesized using the RNA cDNA First Strand Synthesis Kit (Wuhan Servicebio Technology Co., Ltd.; cat. no. G3330), according to the manufacturer's instructions, which included a 50°C incubation step for 60 min with oligo(dT) primers. qPCR analysis was performed on a Bioer fluorescence PCR system using SYBR Green Master Mix (Shanghai Yeasen Biotechnology Co., Ltd.; cat. no. 11201ES). The amplification protocol consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method (51), normalized to GAPDH. The primer sequences were as follows: PINK1 forward, 5'-CCCTCACCCCAACATCATCC-3' and reverse, 5'-ATAACGAGGAACAGCGTCCG-3'; GAPDH forward, 5'-TCCAAAATCAAGTGGGCGA-3' and reverse 5'-AAATGAGCCCCAGCCTTC TC-3'; PGK2 forward, 5'-AACCCAGTGAGACCCCTTTC-3' and reverse 5'-ACCTGAGCGTTCTCGTCA-3'.

Western blot analysis. MCF-7 and MDA-MB-231 breast cancer cells from the OE-NC, PINK1-OE, sh-NC and sh-PINK1 groups were lysed in RIPA buffer (Epizyme; Ipsen Pharma; cat. no. PC101) supplemented with protease inhibitors. Protein concentrations were determined using a BCA kit (Thermo Fisher Scientific, Inc.; cat. no. 23225). Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE (15% for LC3 detection) and transferred to PVDF membranes (MilliporeSigma; cat. no. IPVH00010). The 15% gel improves separation of small LC3 proteins, enabling precise LC3-I/II quantification, key for assessing mitophagy. Larger proteins such as PINK1 and Parkin are well detected on standard gels, so no high-percentage gel was needed. Membranes were blocked with blocking buffer (Epizyme; Ipsen Pharma; cat. no. PS108) for 1 h at room temperature, then incubated overnight at 4°C with the following primary antibodies: PINK1 (1:1,000; Hangzhou HuaAn Biotechnology Co., Ltd.; cat. no. HA723021), Parkin (1:1,000; Hangzhou HuaAn Biotechnology Co., Ltd.; cat. no. HA722952), LC3 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 12741), β -actin (1:5,000; Cell Signaling Technology, Inc.; cat. no. 4970) and PGK2 (1:1,000; Proteintech Group, Inc.; cat. no. 13686-1-AP). After washing, HRP-conjugated secondary antibodies (1:5,000; Proteintech Group, Inc.; cat. no. SA00001-2) were applied for 2 h at room temperature. Signal was visualized using ECL reagent (MilliporeSigma; cat. no. WBKLS0100) and imaged with Image Lab software (version 6.0, Bio-Rad Laboratories, Hercules, Inc.).

Transmission electron microscopy (TEM). Cells from OE-NC, PINK1-OE, sh-NC and sh-PINK1 groups were digested with trypsin and collected at 1×10^6 cells per sample. Following centrifugation at $1,200 \times g$ for 10 min at 4°C, cell pellets were fixed in 2.5% glutaraldehyde (MilliporeSigma; cat. no. G5882) in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. Post-fixation was conducted with 1% osmium tetroxide for 2 h at room temperature to stabilize cellular ultrastructure (notably lipids) and enhance contrast for electron microscopy. Samples were dehydrated sequentially in 50, 70 and 90% ethanol, followed by a 1:1 mixture of 90% ethanol and 90% acetone, then 90% acetone alone. Final dehydration was completed in 100% acetone for 20 min at 4°C. The samples were embedded in epoxy resin and polymerized at 60°C for

24 h. Ultrathin sections (70–90 nm) were prepared with an ultramicrotome (Leica Microsystems GmbH; Model EM UC7) and double-stained with uranyl acetate for 15 min and lead citrate 10 min at 25°C. TEM was performed using a JEOL JEM-1400 transmission electron microscope to assess mitochondrial morphology and autophagosome structures. Digital images were obtained to evaluate the extent of PINK1-mediated mitophagic activity. ImageJ software (National Institutes of Health; version 1.54h), equipped with the JACoP plugin (v2.0), was used for the quantitative analysis of autophagic vesicles in TEM images.

Cell proliferation detection. Cells were seeded in 96-well plates at a density of 2×10^3 cells per well and cultured for 0–96 h. At each designated time point (0, 24, 48, 72 and 96 h), 10 μ l of Cell Counting Kit-8 (CCK-8) reagent (Sangon Biotech Co., Ltd.; cat. no. E606335-500) was added to each well. Following a 2-h incubation period at 37°C, the absorbance at 450 nm was measured using a Bio-Rad Laboratories, Inc. microplate reader. Control wells containing only medium and CCK-8 without cells were included for background subtraction, while peripheral wells were filled with PBS to mitigate edge effects. Proliferation curves were constructed from the absorbance values to evaluate the influence of PINK1 modulation on cellular growth dynamics.

Flow cytometry-based glucose uptake assay. MCF-7 and MDA-MB-231 cells (OE-NC, PINK1-OE and PINK1-OE + siPGK2 groups) were cultured in glucose-free DMEM (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 11966025) containing 10% FBS for 24 h to synchronize metabolic activity. Cells were seeded into 6-well plates at 2×10^5 cells per well. After removing the medium and washing with PBS, 1 ml of 2-NBDG working solution (Beyotime Biotechnology; cat. no. S0561S) was added. Plates were incubated at 37°C for 30 min in the dark. Afterwards, cells were washed twice with PBS and glucose uptake was assessed by flow cytometry (Thermo Fisher Scientific, Inc.; cat. no. A59358) based on 2-NBDG fluorescence. Data were acquired and analyzed using Attune™ Xenith Software (Thermo Fisher Scientific, Inc.; version 1.3). Cells were gated using forward and side scatter to exclude debris and doublets. Fluorescence thresholds were set with unstained control (empty) cells as the baseline and populations exceeding this threshold were defined as positive. All flow cytometry experiments were performed in triplicate (n=3).

Wound healing assay for migration. MCF-7 and MDA-MB-231 breast cancer cells [from the experimental groups OE-NC, PINK1-OE, sh-NC, sh-PINK1 and PINK1-OE combined with the silencing PGK2 (PINK1-OE + siPGK2)] were seeded into 6-well plates that were pre-marked with three parallel reference lines using a sterile ruler. Cells were cultured in DMEM supplemented with 10% FBS until they attained 100% confluency. A 200 μ l pipette tip was utilized to create uniform scratches across the monolayer, intersecting the reference lines for standardized imaging positions. Plates were incubated at 37°C with 5% CO₂ with medium supplemented with 0% FBS, and scratch closure was monitored at 0 and 24 h using an Olympus IX73 microscope (10X objective). ImageJ software

was employed to analyze the residual scratch areas normalized to initial widths, to quantify migration rates.

Transwell invasion assay. Transwell invasion assays were conducted using Matrigel-coated Corning chambers (Corning, Inc.; cat. no. 354480). MCF-7 and MDA-MB-231 cells from the OE-NC, PINK1-OE, sh-NC, sh-PINK1 and PINK1-OE + siPGK2 groups were resuspended in serum-free medium and 1×10^4 cells were seeded into the upper chambers of 24-well plates. The lower chambers were filled with 500 μ l DMEM containing 10% FBS. After 48 h of incubation at 37°C, the inserts were fixed in 4% paraformaldehyde at room temperature for 30 min and stained with crystal violet (Wuhan Servicebio Technology Co., Ltd.; cat. no. G1014) for 30 min at 25°C. Non-invading cells on the upper surface were gently wiped off and invaded cells on the underside were imaged and counted using an Olympus IX71 light microscope to evaluate cell invasion under various conditions.

Targeted metabolite assays. MCF-7 and MDA-MB-231 breast cancer cells (OE-NC, PINK1-OE, sh-NC and sh-PINK1 groups) were lysed in ice-cold RIPA buffer supplemented with protease inhibitors. After centrifugation (12,000 $\times$ g, 15 min, 4°C), supernatants were collected for targeted metabolite assays. PGK2 levels were quantified using the PGK2 ELISA Kit (Jianglai Biotechnology). Specifically, supernatants (50 μ l/well) and biotinylated capture antibodies were incubated in pre-coated 96-well plates for 60 min at 37°C, followed by streptavidin-HRP conjugate incubation and TMB substrate development (15 min in the dark). Pyruvate concentrations were determined via the Pyruvate Colorimetric Assay Kit (Sigma-Aldrich; Merck KGaA; cat. no. MAK567, USA). Specifically, cell lysates (50 μ l) were mixed with 2,4-dinitrophenylhydrazine reagent, incubated at 37°C for 30 min and absorbance was measured at 520 nm. Acetyl-CoA content was assessed using the Acetyl-CoA ELISA Kit (Wuhan Elabscience Biotechnology Co., Ltd.; cat. no. E-BC-F046-48T-ELS). Specifically, samples were diluted 1:5 in assay buffer, transferred to antibody-coated plates with serial standards (0–240 ng/ml), incubated with HRP-conjugated detection antibody (60 min at 37°C) and developed with TMB. Reactions for all assays were terminated with 2 M H₂SO₄ and absorbance at 450 nm (reference 630 nm) was measured using a microplate reader. Total protein normalization was performed via BCA assay, with results expressed as ng/mg protein.

Statistical analysis. All statistical analyses were conducted using SPSS version 18.0 (SPSS, Inc.) and GraphPad Prism 9.0 (Dotmatics). Comparisons between two groups were performed using an unpaired two-tailed Student's t-test, while comparisons among more than two groups were analyzed using one-way ANOVA followed by Tukey's post-hoc test. For non-parametric data, such as differences in enrichment scores between tumor and normal tissues, the Mann-Whitney U test was applied. Spearman's rank correlation coefficient was employed to assess associations between continuous variables. Kaplan-Meier survival curves were generated and compared using the log-rank test. GSEA was conducted to identify significantly enriched pathways. P<0.05 was considered to indicate a statistically significant difference. All data

are presented as the mean $\pm$ standard error of the mean derived from at least three independent experiments.

Results

Differential expression and enrichment in breast cancer. To investigate the transcriptional alterations in breast cancer progression and their relationship with PINK1-mediated mitophagy, a differential expression analysis utilizing TCGA-BRCA RNA-sequencing data was conducted. The analysis identified a total of 12,077 DEGs, comprising 7,995 upregulated and 4,082 downregulated genes. A volcano plot (Fig. S1A) and a heatmap of the top 30 DEGs (Fig. S1B) were generated to visualize the expression patterns. The volcano plot distinctly highlighted the significantly upregulated genes in red and downregulated genes in green, while the heatmap illustrated the expression levels of the top 30 DEGs across samples, with a color gradient representing expression intensity. GO and KEGG enrichment analyses revealed significant enrichment in several classical pathways (Fig. S1C-F). The GO enrichment analysis indicated that upregulated DEGs were significantly associated with biological processes such as cell cycle regulation, chromosomal segregation and organelle fission (Fig. S1C). The most significant terms, based on gene ratios, were ‘organelle fission’ and ‘chromosomal segregation’, with notable enrichment in cell cycle-related processes. Furthermore, structural components such as ‘sarcomeres’, ‘ion channel complexes’ and ‘actin-binding elements’ were significantly enriched (Fig. S1D), underscoring their relevance to mitochondrial dynamics and cellular structure. These processes are closely linked to mitochondrial dynamics and cellular energy regulation, suggesting a potential enhancement of mitophagy.

KEGG pathway analysis indicated notable enrichment in immune-related pathways, particularly ‘cytokine-cytokine receptor interactions’ and ‘viral protein interactions with cytokines’ (Fig. S1E). These immune pathways may influence the tumor microenvironment and interact with mitochondrial function, suggesting a connection to metabolic reprogramming in breast cancer progression. Conversely, metabolic and signaling pathways, including ‘PI3K-Akt signaling’, ‘calcium signaling’ and ‘extracellular matrix-receptor interactions’, were also notably upregulated (Fig. S1F). These pathways are essential for mitochondrial quality control and metabolic reprogramming, indicating that factors such as immune signaling and metabolic pathways may drive metabolic and structural changes by regulating both immune and metabolic signaling pathways. This aligns with prior studies emphasizing the role of mitophagy in tumor progression and energy metabolism, supporting the hypothesis that mitophagy is crucial in breast cancer (52,53). Detailed results on the DEGs and functional enrichment are available in Table S1.

PINK1-mediated mitophagy and immune evasion. Building on the transcriptomic evidence from a PINK1-associated dataset suggesting the enrichment of mitophagy-related pathways in metabolic reprogramming, the clinical and immunological relevance of PINK1 was further investigated using TCGA-BRCA data. The enrichment scores of mitophagy pathways were evaluated using ssGSEA. The findings demonstrated

that several mitophagy-related pathways were upregulated in the tumor group. Notably, the ‘REACTOME_PINK1_PRKN_MEDIATED_MITOPHAGY’ pathway exhibited a significant increase in enrichment scores, whereas the REACTOME_RECEPTOR_MEDIATED_MITOPHAGY pathway displayed no significant changes (Fig. 1A-D). These results suggest that PINK1-mediated mitophagy may be particularly active in tumor tissues, potentially facilitating tumor progression through metabolic reprogramming. The absence of significant changes in receptor-mediated mitophagy pathways implies a more specific role for PINK1 in this context. In-depth prognostic analysis of mitophagy pathway enrichment scores revealed that higher enrichment in several pathways was associated with poorer patient outcomes (Fig. 1E and F). To avoid potential violation of the proportional hazards assumption caused by late-stage crossover of survival curves in Fig. 1F, survival analysis was restricted to data collected up to 2018 and statistical significance was evaluated using the log-rank test. Consistent with this, survival analysis indicated that samples with high PINK1 expression were associated with a significantly poorer survival (Fig. 1G). The differential mitochondrial autophagy-related genes and pathway enrichment scores are provided in Tables SII and SIII.

Subsequently, the relationship between PINK1 expression and the infiltration scores of 22 immune cell types were calculated via the CIBERSORT algorithm. PINK1 expression demonstrated significant correlations with several immune cell types, including CD8 T cells, activated dendritic cells, memory B cells and M0 macrophages (Fig. 1H). Notably, PINK1 showed significant associations with both M1 and M2 macrophage infiltration, with a markedly stronger correlation for M2 macrophages. This aligns with, rather than challenges, its canonical role in M2 polarization across different cancer types, while also hinting at context-specific patterns that may modulate the strength of these associations (54).

Moreover, the correlations between PINK1 expression and the expression of characteristic genes of CAFs, M1 macrophages and M2 macrophages were analyzed. In CAFs, PINK1 exhibited the strongest positive correlations with PDGFRB, BHLHE40 and COL3A1, key genes involved in CAF-mediated extracellular matrix remodeling and tumor progression (Fig. S2A-C). In M1 macrophages, the highest negative correlations were observed with CXCL10, IDO1 and CXCL11, which are markers of inflammatory response and immune activation (Fig. S2D-F). Conversely, in M2 macrophages, PINK1 demonstrated the highest positive correlation with TGFBI, IL33 and MRC1, markers that contribute to immune suppression and tissue remodeling (Fig. S2G-I). These findings underscore the potential role of PINK1 in modulating both immune responses and the tumor microenvironment, further supporting its dual role in breast cancer progression.

Collectively, these results support a dual-role model for PINK1, indicating that it may not only facilitate metabolic adaptation through mitophagy but may also contribute to immune evasion, both of which play critical roles in promoting breast cancer progression.

PINK1 high expression alters the immune-related transcriptome. Building on evidence that PINK1-driven mitophagy may promote metabolic remodeling in breast

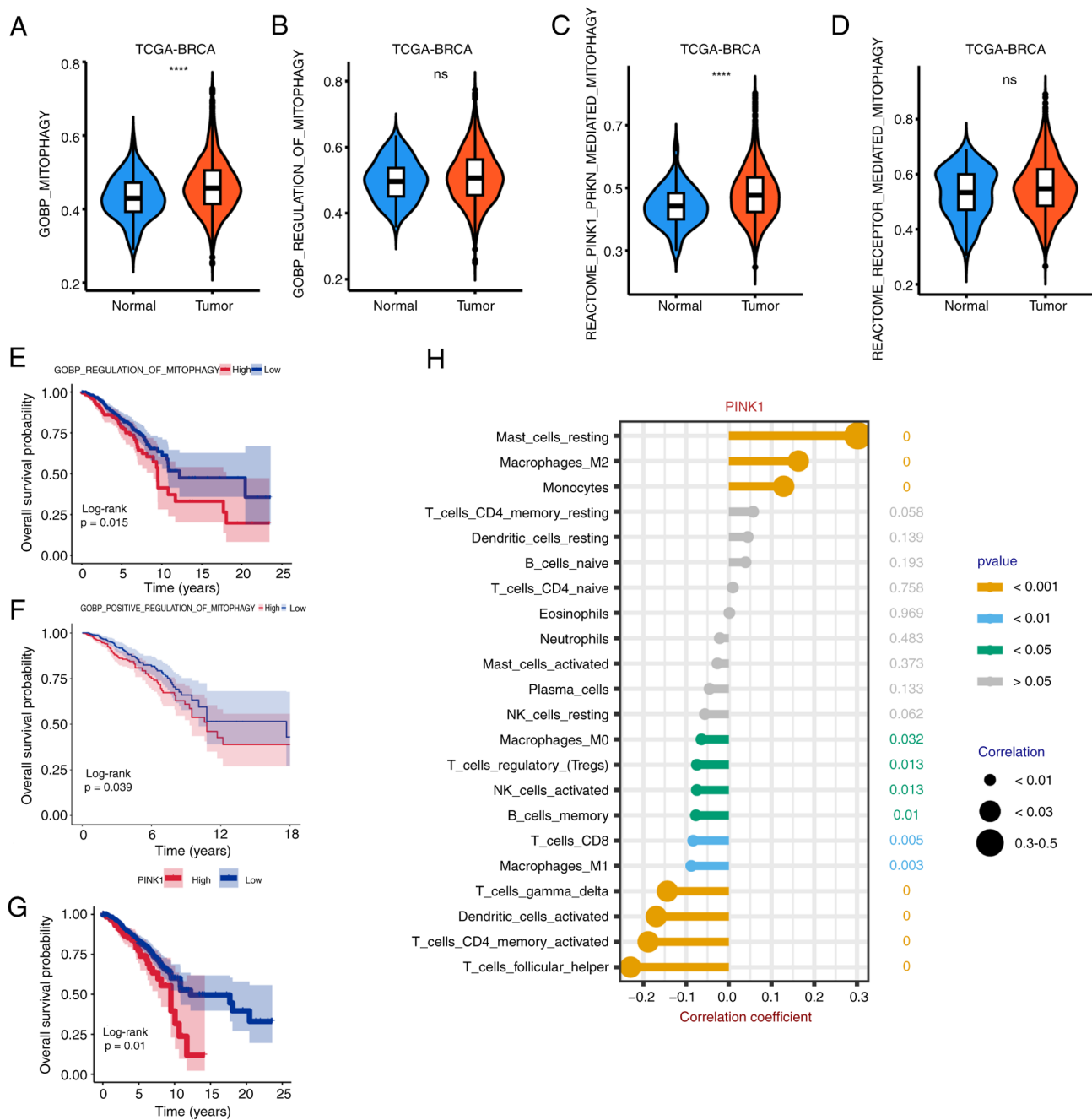


Figure 1. Evaluation of mitophagy activity, prognostic relevance and immune correlation of PINK1 in breast cancer. Violin plots depicting enrichment scores of the (A) GOBP_MITOPHAGY, (B) GOBP_REGULATION_OF_MITOPHAGY, (C) REACTOME_PINK1_PARKIN_MEDIATED_MITOPHAGY and (D) REACTOME_RECEPTOR_MEDIATED_MITOPHAGY gene sets in tumor and normal tissues from TCGA-BRCA (Mann-Whitney U test). Prognostic analysis of mitochondrial autophagy pathway enrichment scores of the (E) GOBP_REGULATION_OF_MITOPHAGY and (F) GOBP_POSITIVE_REGULATION_OF_MITOPHAGY gene sets. (G) Clinical prognosis analysis of PINK1 (log-rank test). (H) Correlation analysis between PINK1 and the immune cell infiltration scores of 22 types calculated by CIBERSORT (Spearman's rank correlation analysis). Data are represented as the mean $\pm$ SEM from at least three independent experiments. **** $P < 0.0001$. PINK1, PTEN-induced kinase 1; TCGA, The Cancer Genome Atlas; BRCA, Breast Invasive Carcinoma; ns, not significant.

cancer, the transcriptomic changes associated with high PINK1 expression were investigated. The DEGs between PINK1-high and PINK1-low samples were analyzed utilizing TCGA-BRCA data. As illustrated in Fig. S3A, the volcano plot revealed a significant cohort of DEGs, with red and green dots representing significantly upregulated and downregulated genes, respectively. The corresponding heatmap (Fig. S3B) highlights the top DEGs, with hierarchical clustering clearly distinguishing the PINK1-high and PINK1-low groups at the

transcriptome level, showcasing distinct expression patterns between the two groups. Functional enrichment analyses were performed on the downregulated genes. GO biological process terms (Fig. S3C) were predominantly related to immune responses, including 'immunoglobulin production', 'humoral immune activity' and 'bacterial defense mechanisms'. The most enriched terms, with significant P-values, also included 'immune regulation' and 'antigen processing', underscoring the potential immunosuppressive roles of PINK1 in breast

cancer progression. Cellular component analysis (Fig. S3D) revealed decreased expression in ‘immunoglobulin complexes’ and ‘blood microparticles’. Molecular function enrichment (Fig. S3E) demonstrated significant reductions in ‘antigen binding’, ‘receptor activity’ and ‘neurotransmitter-related functions’. These findings suggest that high PINK1 expression may contribute to immune evasion by downregulating key immune functions such as antigen recognition and receptor signaling, thereby facilitating breast cancer progression. Consistent with these findings, KEGG pathway enrichment (Fig. S3F) identified several suppressed signaling pathways, including ‘neuroactive ligand-receptor interaction’, ‘olfactory’ and ‘taste transduction’, and immune-related processes such as ‘Neutrophil extracellular trap formation’. These results indicate that high PINK1 expression fosters an immunosuppressive transcriptional landscape, potentially aiding immune evasion during tumor progression. The differential analysis and functional enrichment results of PINK1 single-gene analysis are provided in Table SIV.

PINK1-mediated mitophagy activation. Previous transcriptional and survival analyses suggested that PINK1-mediated mitophagy may contribute to breast cancer progression through metabolic reprogramming. To validate this mechanism, PINK1 was overexpressed in both luminal MCF-7 and triple-negative MDA-MB-231 cells. In MCF-7 cells, RT-qPCR analysis demonstrated that PINK1 overexpression resulted in a robust upregulation increase in PINK1 mRNA levels compared with the vector control group (Fig. 2A), thereby confirming effective transcriptional upregulation. Western blot analysis further indicated a marked increase in PINK1 protein levels, in conjunction with the elevated expression of its downstream mitophagy-related targets, Parkin and LC3-II (Fig. 2B). Due to its low molecular weight, LC3 was resolved on 15% SDS-PAGE gels, with β -actin serving as the corresponding loading control (Fig. 2B and D). Densitometric analysis (Fig. 2E) corroborated the statistically significant increase in all proteins, signifying activation of the PINK1-Parkin-mediated mitophagy pathway. Similar results were observed in MDA-MB-231 cells, where PINK1 overexpression significantly increased mRNA levels (Fig. 2C) and markedly increased the protein levels of PINK1, Parkin and LC3-I/II (Fig. 2D). Semi-quantitative analysis (Fig. 2F) further validated the upregulation of these markers, indicating conserved activation of mitophagy across both luminal and TNBC subtypes.

Notably, the elevated levels of LC3-II observed in both cell lines suggest not only enhanced mitophagosome formation but also increased autophagic flux. These findings provide further molecular evidence that PINK1 serves as a crucial regulator of mitochondrial quality control in breast cancer. By facilitating Parkin-mediated mitophagy, PINK1 may contribute to mitochondrial quality control in breast cancer cells.

Ultrastructural evidence of mitophagy activation. Following biochemical validation of PINK1-induced mitophagy, TEM was employed to investigate the ultrastructural changes in MCF-7 and MDA-MB-231 breast cancer cells, with the objective of determining whether PINK1 overexpression promotes mitophagy at the organelle level. Representative TEM images (Fig. 3) illustrated that PINK1-OE breast cancer cells

contained numerous double-membraned autophagosomes (indicated by yellow arrows), frequently associated with damaged mitochondria, thereby indicating active mitophagy. Conversely, control cells display fewer autophagic vesicles, predominantly intact mitochondria and limited interaction between autophagosomes and mitochondria, suggesting a low basal level of mitophagy. TEM images reveal an apparent increase in autophagic vesicles in PINK1-OE cells, indicating enhanced mitochondrial clearance (Fig. 3). Mitochondrial remnants were also observed within autophagosomes. This finding, supported by the co-localization of mitochondrial and autophagosomal markers, provides evidence of selective mitophagy as opposed to non-specific autophagy. These morphological findings underscore that PINK1 may enhance mitochondrial clearance in both luminal and TNBC cells. By promoting mitophagy, PINK1 may facilitate cellular metabolic reprogramming under stress by preserving mitochondrial function and energy production, thereby aiding tumor cell survival and proliferation amid metabolic challenges.

PINK1 overexpression accelerates breast cancer cell migration. Having established that PINK1 may activate mitophagy and enhance mitochondrial quality, whether this activity increases tumor aggressiveness was subsequently investigated. PINK1 was overexpressed in luminal MCF-7 and triple-negative MDA-MB-231 cells, followed by cell viability and migration assays. In MCF-7 cells, PINK1 overexpression resulted in a progressive increase in cell viability over time. The CCK-8 assay (Fig. 4A) demonstrated that PINK1-OE cells exhibited enhanced proliferation from 24 to 96 h, with statistically significant differences noted at the 96-h mark (Fig. 4B). This proliferative advantage suggests that PINK1 contributes to the long-term growth potential of luminal breast cancer cells. Similarly, MDA-MB-231 cells also displayed a significant increase in viability upon PINK1-OE at 96 h (Fig. 4C and D), although the magnitude of change was less pronounced than in MCF-7 cells.

To further assess whether PINK1 influences the migratory behavior of breast cancer cells, wound healing assays were conducted. As illustrated in Fig. 4E and G, wound closure was significantly accelerated in both PINK1-OE MCF-7 and MDA-MB-231 cells after 24 h, compared with the vector-transfected controls. Quantitative analysis of the wound area revealed an almost 1.68-fold increase in migration distance for MCF-7 cells (Fig. 4F) and a significant increase for MDA-MB-231 cells (Fig. 4H). These findings demonstrate that PINK1 overexpression promoted both the proliferation and migration of breast cancer cells, extending its functional role beyond the activation of mitophagy. The enhanced migration may be associated with metabolic rewiring induced by PINK1-mediated mitochondrial clearance, which supports tumor cell survival and adaptation to metabolic stress.

PINK1 enhances the invasive potential of breast cancer cells. To further elucidate the implications of PINK1-mediated mitophagy and metabolic remodeling, it was investigated whether PINK1 promotes breast cancer cell invasion. Matrigel-coated Transwell assays were conducted to assess the invasive capacity of both luminal MCF-7 and triple-negative MDA-MB-231 cells. Representative images revealed that both

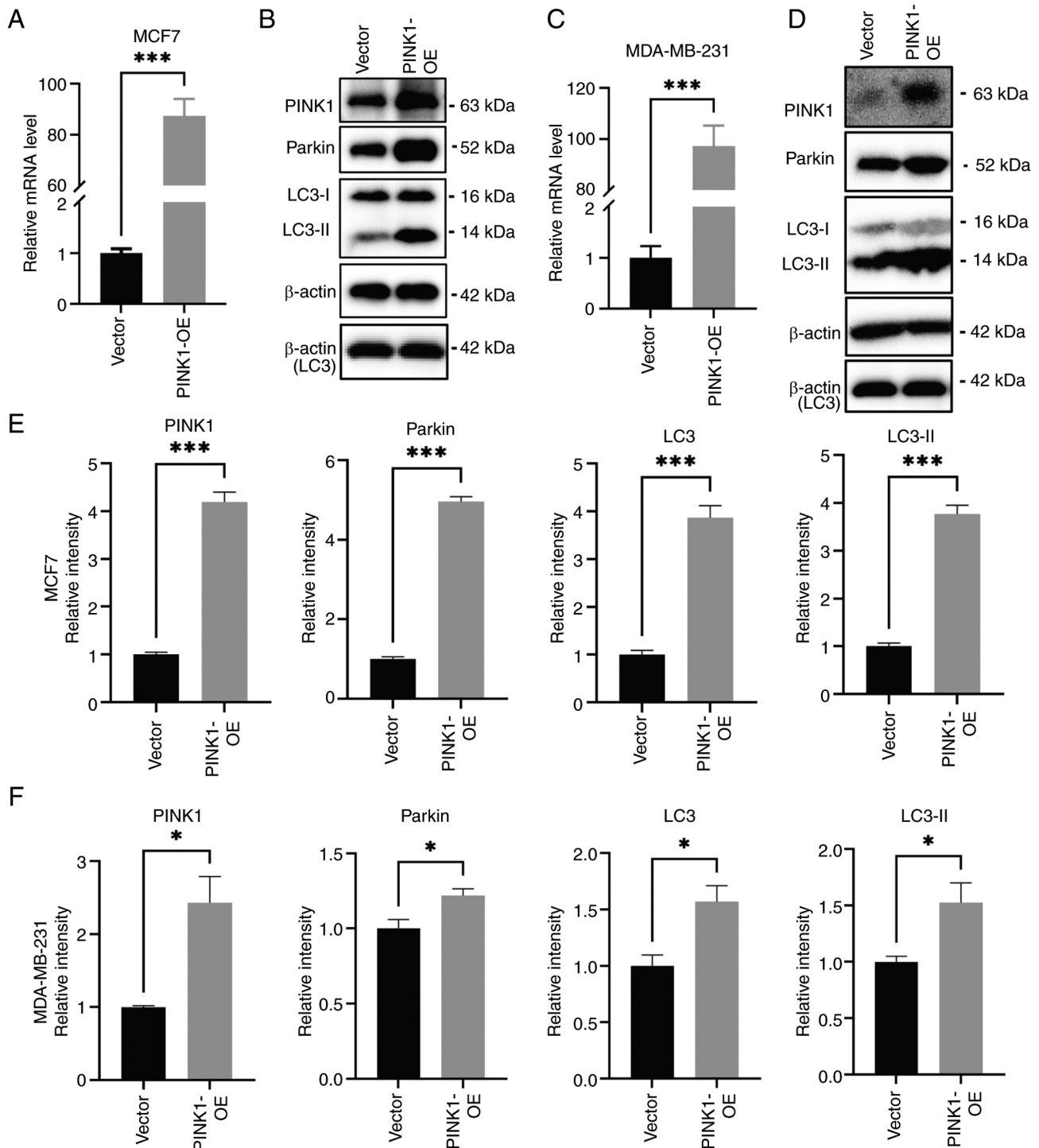


Figure 2. Analysis of PINK1 overexpression and its impact on mitophagy markers in breast cancer cells. (A) RT-qPCR analysis was used to measure PINK1 mRNA expression in MCF-7 cells transfected with either a control vector or a PINK1 overexpression plasmid. (B) Western blot analysis was performed to assess the protein levels of PINK1, Parkin and LC3-I/II in MCF-7 cells. (C) RT-qPCR analysis was used to measure PINK1 mRNA expression in MDA-MB-231 cells transfected with either a control vector or a PINK1 overexpression plasmid. (D) Western blot analysis was performed to assess the protein levels of PINK1, Parkin and LC3-I/II in MDA-MB-231 cells. Densitometric quantification of the indicated proteins, normalized to β -actin, is shown for (E) MCF-7 and (F) MDA-MB-231 cells. The β -actin band labeled as 'LC3' denotes the loading control for the LC3 experimental condition and was separated on 15% SDS-PAGE; all other samples were separated on 10% SDS-PAGE. Statistical analysis was performed using an unpaired two-tailed Student's t-test. Data are represented as the mean $\pm$ SEM from at least three independent experiments. * $P < 0.05$, *** $P < 0.001$. RT-qPCR, reverse transcription-quantitative PCR; PINK1, PTEN-induced kinase 1; OE, overexpression.

MCF-7 and MDA-MB-231 cells overexpressing PINK1 exhibited a marked increase in the number of blue-stained cells that penetrated the Matrigel barrier compared with their respective vector control groups (Fig. 5A and B). The cells were stained

with crystal violet to visualize and quantify invasion, with the number of invaded cells counted across multiple fields of view. This increase in invasive cells was consistently observed across multiple replicates, indicating that PINK1 significantly

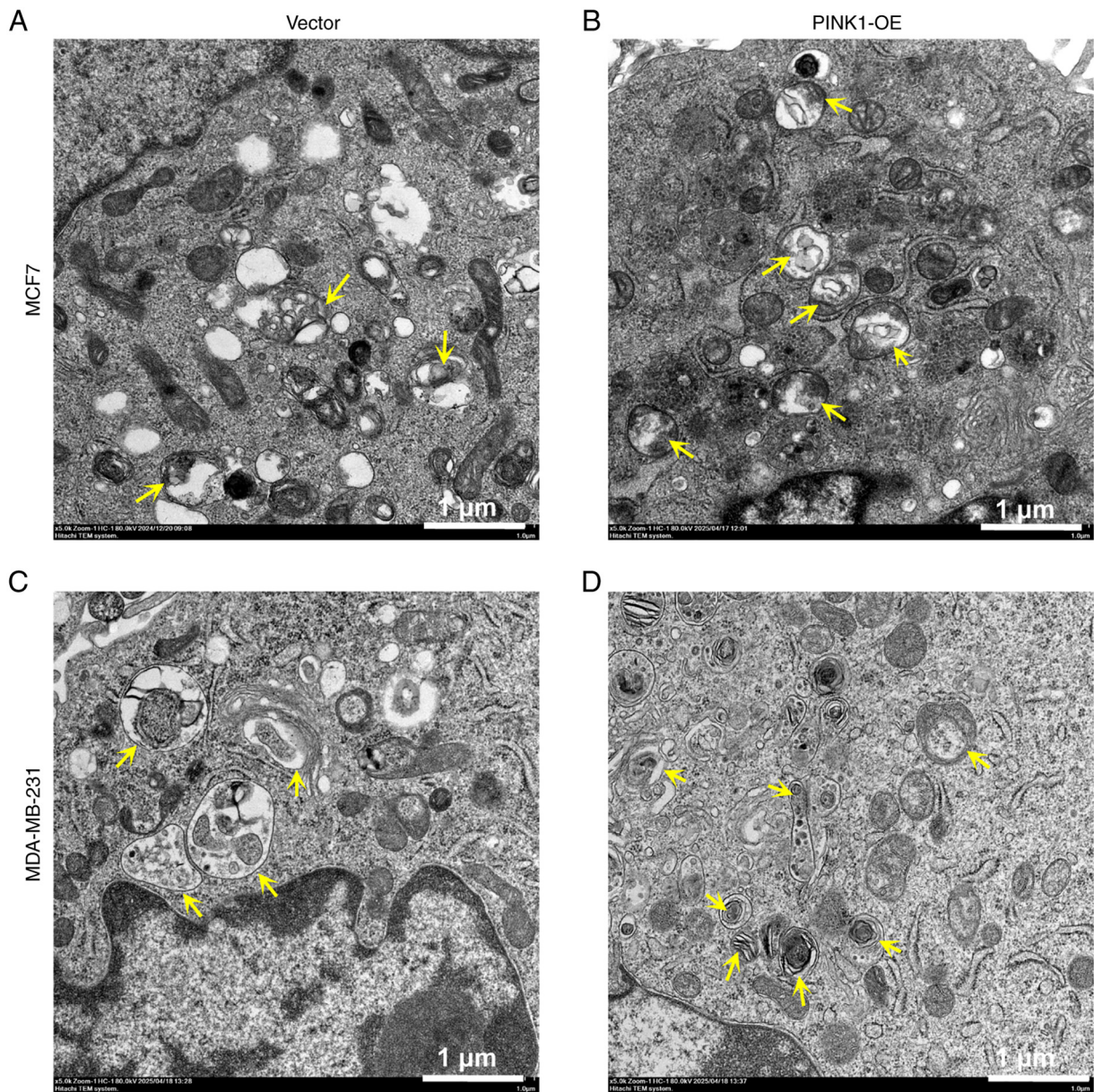


Figure 3. Ultrastructural analysis of PINK1-regulated mitophagy in breast cancer cells. TEM images of MCF-7 cells transfected with either (A) vector control or (B) PINK1 overexpression plasmid. TEM images of MDA-MB-231 cells with (C) vector control or (D) PINK1 overexpression plasmid. The yellow arrows indicate autophagic structures formed during mitophagy. PINK1, PTEN-induced kinase 1; TEM, transmission electron microscopy; OE, overexpression.

enhanced cellular invasiveness. Quantitative analysis of the invaded cells demonstrated that PINK1-OE induced approximately a 2-fold increase in invasive capacity in MCF-7 cells compared with vector controls (Fig. 5C). In MDA-MB-231 cells, which are inherently more invasive, PINK1-OE also led to a significant increase in invasion (Fig. 5D), reinforcing the pro-invasive role of PINK1 across different molecular subtypes of breast cancer. These findings indicate that PINK1 overexpression markedly enhanced the invasive potential in both luminal and TNBC subtypes. Beyond its established role in mitophagy, PINK1 emerges as a key regulator of tumor cell invasion. These findings indicate that PINK1 overexpression markedly enhanced the invasive potential in both luminal (MCF-7) and TNBC (MDA-MB-231) subtypes, reinforcing its pro-invasive role across different breast cancer molecular subtypes.

Knocking down PINK1 expression inhibits tumor growth. The efficacy of knockdown in MCF-7 and MDA-MB-231 cells was confirmed through RT-qPCR and western blot analysis. The qPCR results demonstrated a significant reduction in PINK1 mRNA levels in MCF-7 cells (Fig. 6A) compared with the control, thereby confirming effective knock down. Western blot analysis further corroborated the downregulation of PINK1 protein expression (Fig. 6B), which was associated with notable reductions in Parkin and the autophagy marker LC3-II (Fig. 6E). These findings imply that PINK1 knockdown disrupted the mitophagy machinery, as evidenced by decreased Parkin expression and a lower LC3-II/LC3-I ratio, a well-established indicator of autophagosome maturation (55). In MDA-MB-231 cells, a more aggressive subtype of TNBC, a similar pattern was observed. PINK1 mRNA was significantly downregulated

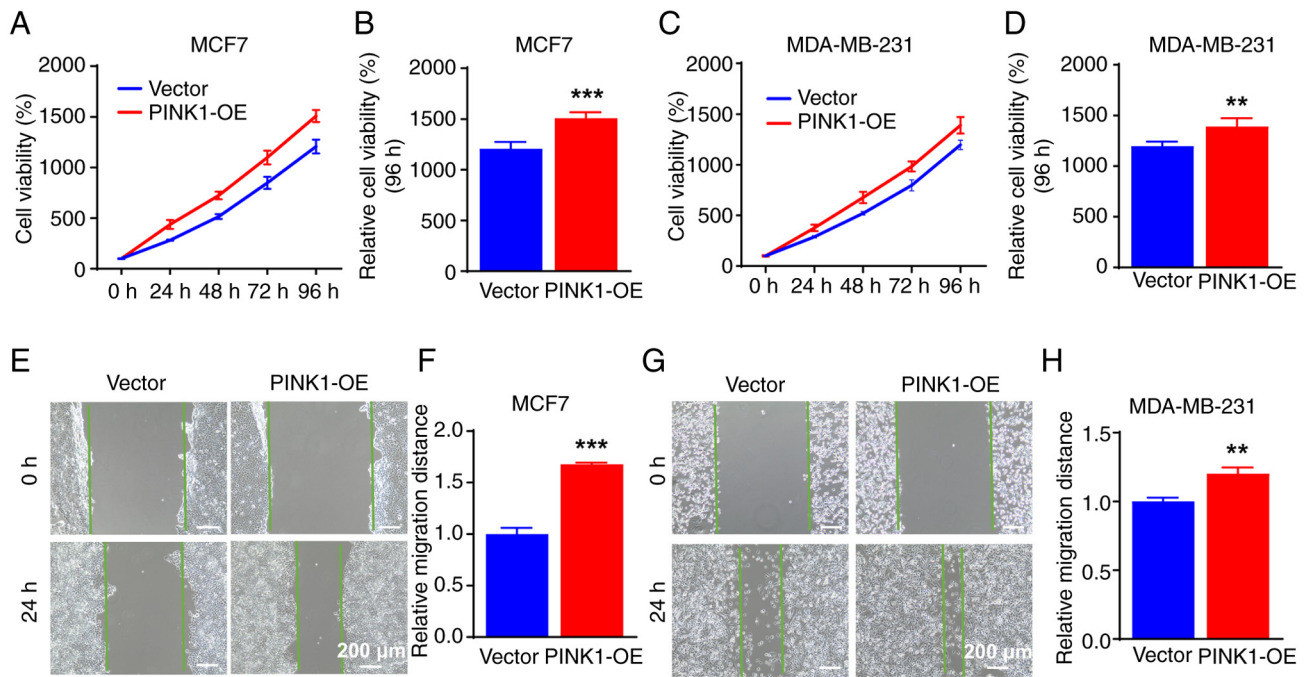


Figure 4. Functional assessment of PINK1 overexpression in breast cancer cell proliferation and migration. (A) Cell viability curve of MCF-7 cells transfected with either vector control or PINK1 overexpression plasmid over 96 h. (B) Quantitative analysis of MCF-7 cell viability at 96 h. (C) Cell viability curve of MDA-MB-231 cells under the same conditions. (D) Quantitative analysis of MDA-MB-231 viability. (E) Wound healing images and (F) quantification of the migration ability of MCF-7 cells at 0 and 24 h. (G) Wound healing images and quantification of (H) the migration ability of MDA-MB-231 cells at 0 and 24 h. Statistical analysis was performed using an unpaired two-tailed Student's t-test. Data are represented as the mean $\pm$ SEM from at least three independent experiments. ** P <0.01, *** P <0.001. PINK1, PTEN-induced kinase 1; OE, overexpression.

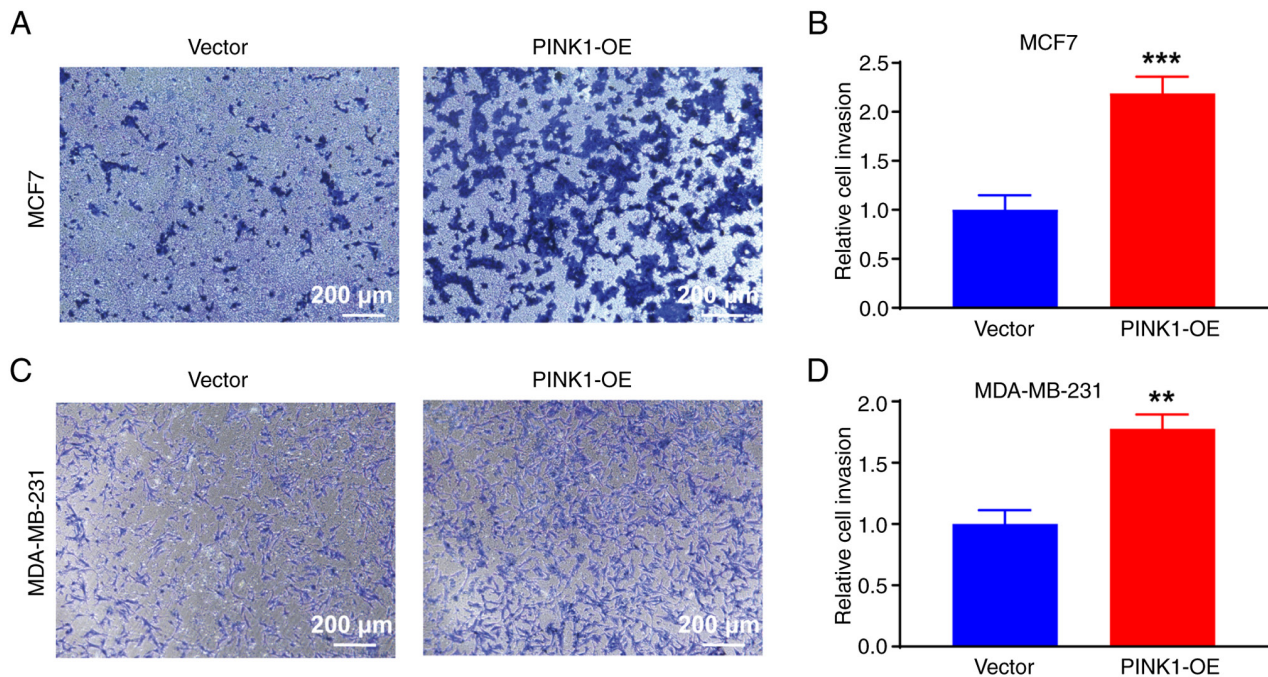


Figure 5. Transwell invasion assay illustrating the effect of PINK1 overexpression on breast cancer cell invasiveness. (A) Representative Transwell invasion images and (B) quantification of the number of invasive MCF-7 cells transfected with control vector or PINK1 overexpression plasmid. (C) Representative Transwell invasion images and (D) quantification of the number of invasive MDA-MB-231 cells transfected with control vector or PINK1 overexpression plasmid. Statistical analysis was performed using an unpaired two-tailed Student's t-test. Data represent means $\pm$ SEM from at least three independent experiments. ** P <0.01, *** P <0.001. PINK1, PTEN-induced kinase 1; OE, overexpression.

following shRNA-mediated knockdown (Fig. 6C), consistent with the results obtained in MCF-7 cells. Protein analysis revealed consistent reductions in PINK1, Parkin and LC3-II

(Fig. 6D and F), thereby validating the findings in a second breast cancer cell model. Reduced LC3-II indicates blocked autophagosome maturation and impaired mitophagy. The

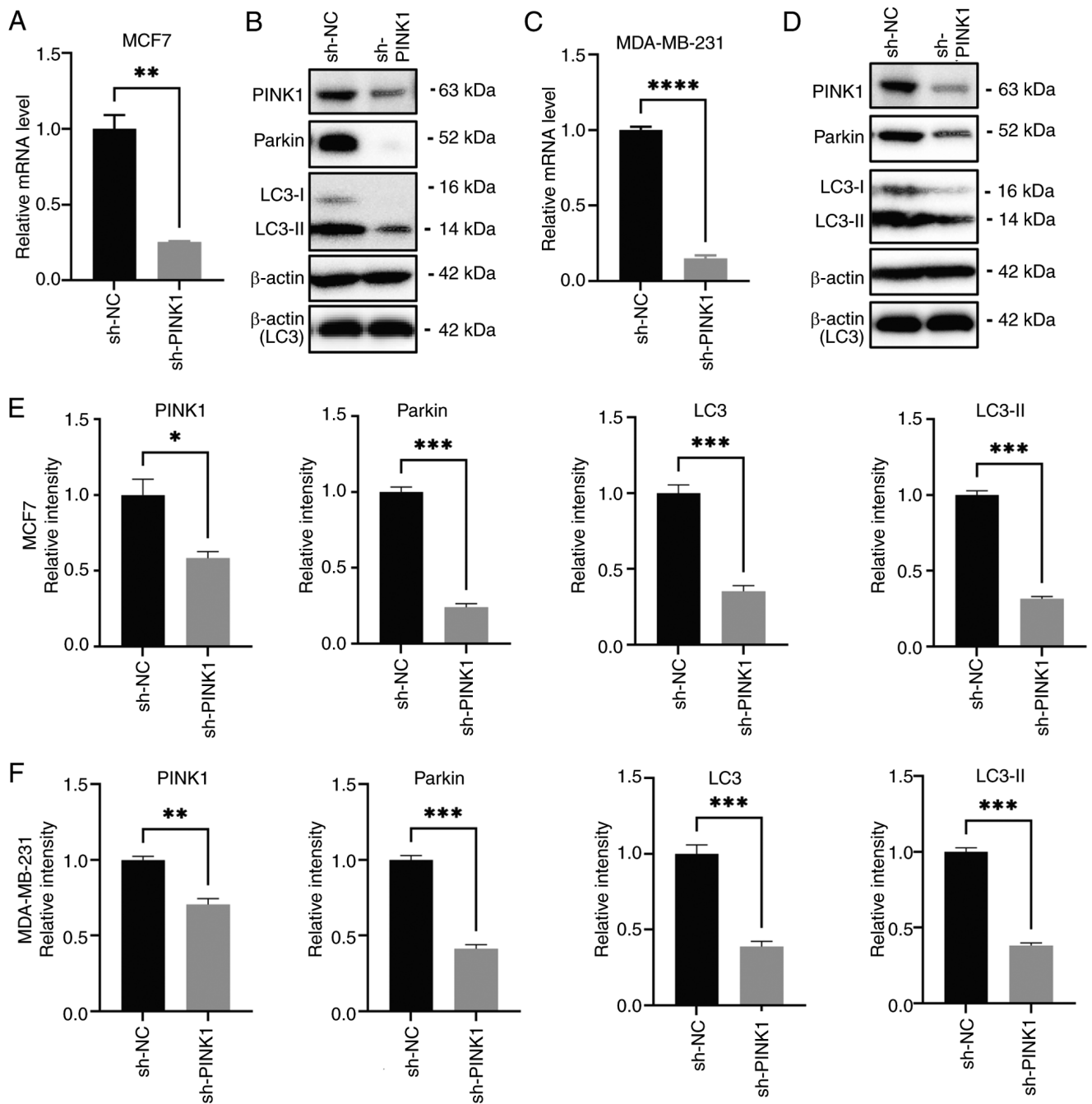


Figure 6. Analysis of PINK1 knockdown and its impact on mitophagy-related proteins in breast cancer cells. (A) Relative mRNA levels of PINK1 in MCF-7 cells transfected with shRNA targeting PINK1 or a negative control were measured by quantitative PCR. (B) Western blot analysis displayed PINK1, Parkin and LC3-I/II protein levels in MCF-7 cells following PINK1 knockdown. (C) Relative mRNA levels of PINK1 in MDA-MB-231 cells transfected with shRNA targeting PINK1 or a negative control were measured by quantitative PCR. (D) Western blot analysis displayed PINK1, Parkin and LC3-I/II protein levels in MDA-MB-231 cells following PINK1 knockdown. Densitometric quantification of PINK1, Parkin and LC3-I/II protein expression in (E) MCF-7 and (F) MDA-MB-231 cells is also presented. The β-actin band labeled as 'LC3' denotes the loading control for the LC3 experimental condition and was separated on 15% SDS-PAGE; all other samples were separated on 10% SDS-PAGE. Statistical analysis was performed using an unpaired two-tailed Student's t-test. Data are represented as the mean ± SEM from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. PINK1, PTEN-induced kinase 1; sh, short hairpin; NC, negative control.

persistent attenuation of both mitophagy markers across two distinct breast cancer cell lines suggests a conserved role for PINK1 in maintaining mitochondrial quality control. These results indicate that PINK1 may be essential for sustaining mitophagic flux and mitochondrial homeostasis; its depletion disrupts the PINK1/Parkin axis, inhibiting the autophagic clearance of dysfunctional mitochondria.

Ultrastructural evidence of mitophagy suppression. TEM was employed to examine mitophagy at the ultrastructural level in breast cancer cells following PINK1 knockdown. In control MCF-7 cells (Fig. 7A), numerous double-membrane autophagosomes and autolysosomes containing degraded mitochondria were observed, indicating active mitophagy. Quantitative analysis revealed PINK1-knockdown MCF-7

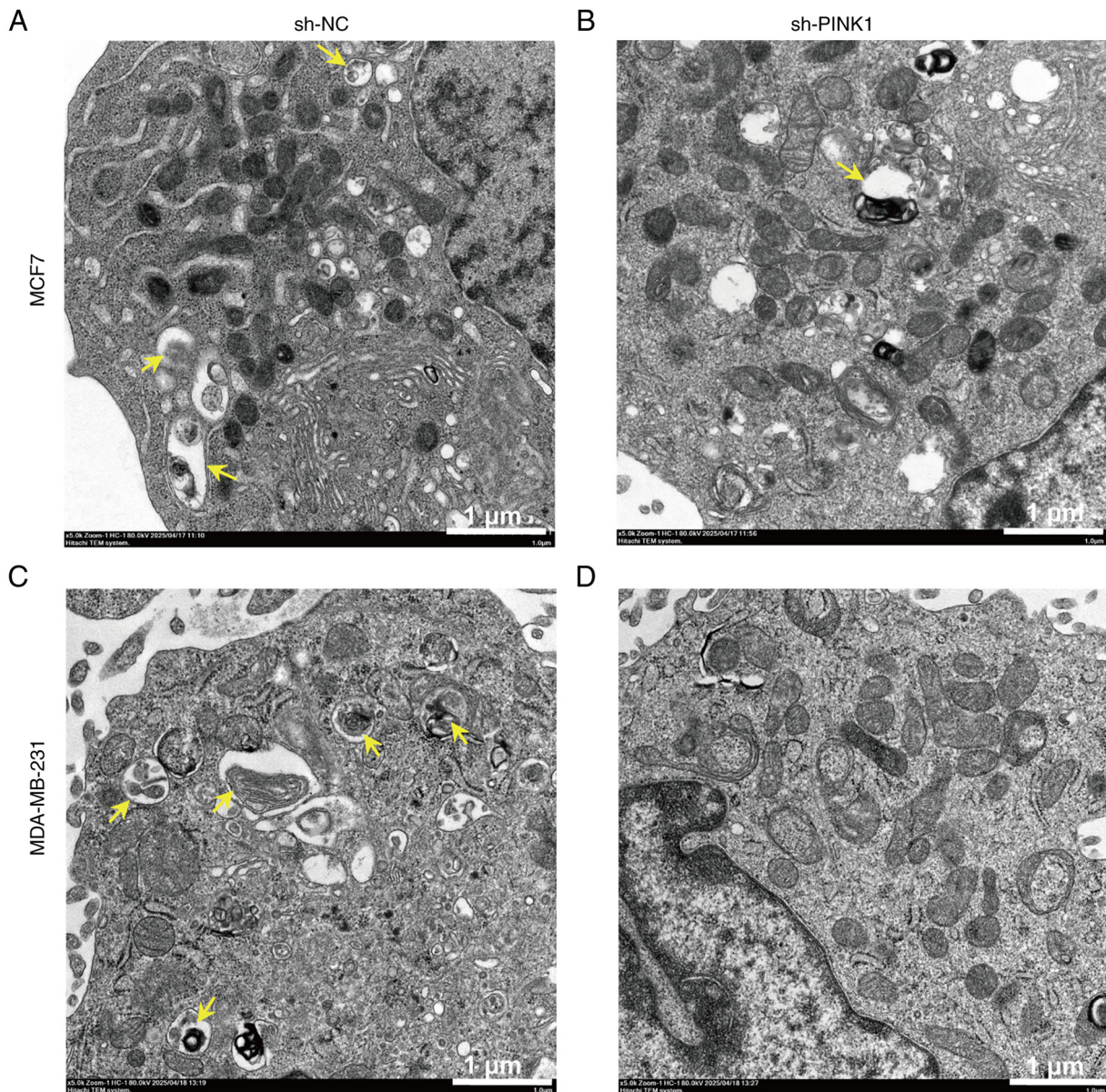


Figure 7. Ultrastructural assessment of PINK1-induced mitophagy in breast cancer cells. TEM images of MCF-7 cells transfected with either (A) sh-NC or (B) sh-PINK1 groups. TEM images of MDA-MB-231 cells transfected with either (C) sh-NC or (D) sh-PINK1 groups. The yellow arrows indicate autophagic structures formed during mitophagy. PINK1, PTEN-induced kinase 1; TEM, transmission electron microscopy; sh, short hairpin; NC, negative control.

cells exhibited a marked reduction in mitophagic structures accompanied by an accumulation of morphologically intact mitochondria in the cytoplasm, compared with the control group (Fig. 7A and B). This suggests a failure in the mitophagy process, leading to impaired mitochondrial turnover. A similar pattern was noted in MDA-MB-231 cells, where the control group (Fig. 7C) revealed visible mitophagosomes containing mitochondria. Qualitative assessment of electron micrographs suggested a reduction in mitophagic structures in PINK1-knockdown cells (Fig. 7C and D). These ultrastructural differences provide morphological evidence that PINK1 may be critical for mitophagosome formation and mitochondrial clearance in both luminal and TNBC cells. The impairment in mitophagy associated with PINK1 knockdown may disrupt mitochondrial turnover. This leads to an accumulation of dysfunctional mitochondria, which compromises metabolic homeostasis and cellular adaptation to metabolic stress. Collectively, these

findings suggest that PINK1-driven mitophagy promotes breast cancer progression by preserving mitochondrial integrity and facilitating metabolic reprogramming.

PINK1 knockdown inhibits breast cancer cell proliferation and migration. Building on previous evidence that PINK1 enhanced mitophagy, the effects of its depletion on fundamental cellular behaviors in breast cancer were subsequently investigated. Utilizing established stable knockdown models of MCF-7 and MDA-MB-231 cells, alterations in proliferation and migration capacity were assessed. Cell viability was evaluated using the CCK-8 assay over a 96-h period. In MCF-7 cells, PINK1 knockdown markedly decreased viability compared with the negative control, with the difference becoming more pronounced over time (Fig. 8A). By 96 h, the reduction in cell viability was statistically significant (Fig. 8B). A similar trend was observed in MDA-MB-231 cells, although the inhibitory

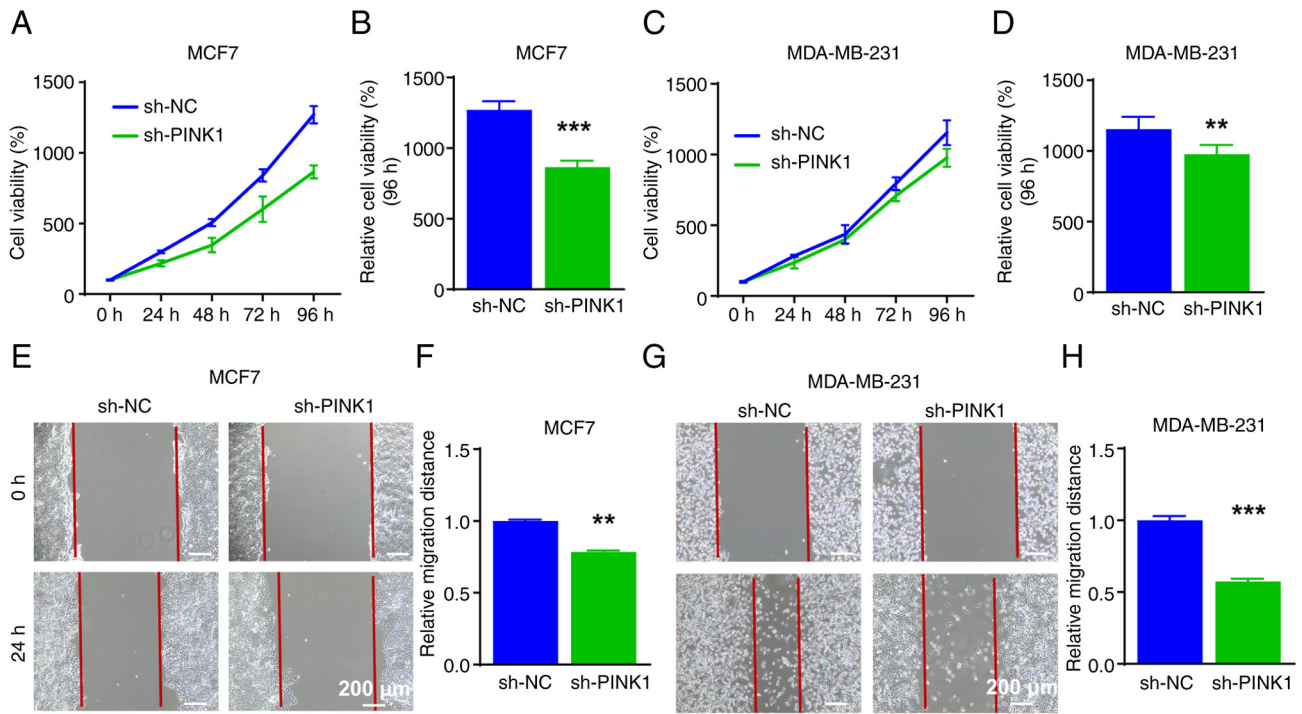


Figure 8. Functional assessment of PINK1 knockdown on breast cancer cell proliferation and migration. (A) Cell viability curves and (B) 96 h statistical comparison of MCF-7 cells transfected with shRNA targeting PINK1 or a negative control. (C) Cell viability curves and (D) 96 h statistical comparison of MDA-MB-231 cells transfected with shRNA targeting PINK1 or a negative control. (E) Representative wound healing images and (F) corresponding migration quantification of the migratory capacity of MCF-7 cells after PINK1 knockdown at 0 and 24 h. (G) Representative wound healing images and (H) quantification of the migratory capacity of MDA-MB-231 cells at 0 and 24 h. Statistical analysis was performed using an unpaired two-tailed Student's t-test for pairwise comparisons. Data are presented as the mean $\pm$ SEM from at least three independent experiments. ** $P < 0.01$, *** $P < 0.001$. PINK1, PTEN-induced kinase 1; sh, short hairpin; NC, negative control.

effect was relatively modest; viability at 96 h was still significantly diminished (Fig. 8C and D). Wound-healing assays were conducted to evaluate the impact of PINK1 knock down on cellular migration. The migration of MCF-7 cells was significantly impaired after 24 h of PINK1 knockdown, a finding corroborated in MDA-MB-231 cells, where a noticeable reduction in wound closure was also observed (Fig. 8E-H).

These results demonstrated that PINK1 may be essential not only for maintaining mitochondrial quality via mitophagy but also for supporting the proliferative and migratory capacities of breast cancer cells. PINK1 knockdown in MCF-7 and MDA-MB-231 cells led to a reduction in cell viability over time and impaired wound closure. These findings indicate that PINK1 plays a role in maintaining basic cellular health in breast cancer cells.

PINK1 knockdown impairs the invasive capacity of breast cancer cells. Previous findings established that PINK1 may promote breast cancer cell proliferation and migration, possibly through mitophagy-mediated metabolic reprogramming. To further evaluate how PINK1 influences the invasive capacity of breast cancer cells, Matrigel-coated Transwell invasion assays were conducted. As illustrated in Fig. 9A, PINK1 knockdown in MCF-7 cells reduced the number of crystal violet-stained cells that invaded the Matrigel layer compared with the negative control. Quantitative analysis confirmed this significant decrease in invasion (Fig. 9C). Similarly, in the highly invasive MDA-MB-231 cells, PINK1 knockdown also resulted in a significant decline in the number of invasive

cells (Fig. 9B and D). PINK1 knockdown significantly reduced the number of cells invading through the Matrigel in both the MCF-7 and MDA-MB-231 cell line models. These results indicate that PINK1 may contribute to the invasive capacity of breast cancer cells.

PINK1 activates PGK2 to link mitophagy with glycolytic reprogramming. To investigate the metabolic effects of PINK1-mediated mitophagy on glycolysis in breast cancer, bioinformatics alongside experimental validation was utilized. An intersection analysis between mitophagy-related genes and DEGs in breast cancer identified 4 significantly differentially expressed overlapping genes (Fig. 10A). GSEA of the glycolysis pathway in cancerous and adjacent tissues revealed a trend toward upregulation of the HALLMARK_GLYCOLYSIS pathway in the Tumor group, although the P-value was not statistically significant (Fig. 10B). Prognostic analysis of glycolysis pathway enrichment scores indicated that higher enrichment was associated with a poorer patient prognosis, as determined by Kaplan-Meier survival analysis and log-rank tests (Fig. 10C). Correlation analysis revealed a strong positive relationship between mitophagy and glycolysis pathway enrichment scores. Notably, the REACTOME_PINK1_PRKN_MEDIATED_MITOPHAGY pathway showed high correlation with several glycolysis-related pathways (Fig. 10D-F). Based on these results, the correlation between this pathway and differential glycolysis genes was further analyzed, highlighting those with a positive correlation coefficient > 0 (Fig. 10G). The differential Glyco-DEGs, pathway

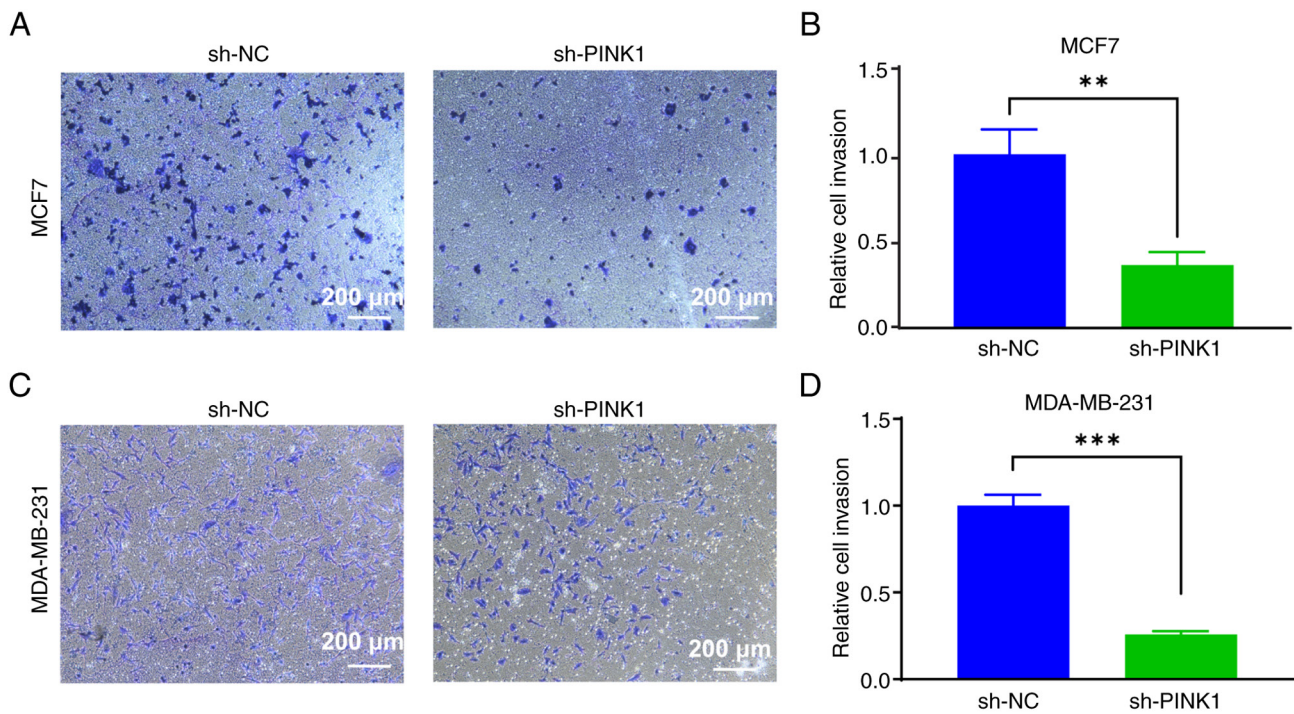


Figure 9. Transwell invasion assay evaluating the impact of PINK1 knockdown on breast cancer cell invasiveness. (A) Representative Transwell invasion images and (B) quantitative analysis of the number of invasive MCF-7 cells transfected with shRNA targeting PINK1 or negative control. (C) Representative Transwell invasion images and (D) quantitative analysis of the number of invasive MDA-MB-231 cells transfected with shRNA targeting PINK1 or negative control. Statistical analysis was performed using a unpaired two-tailed Student's t-test. Data are represented as the mean $\pm$ SEM from at least three independent experiments. ** $P < 0.01$, *** $P < 0.001$. PINK1, PTEN-induced kinase 1; sh, short hairpin; NC, negative control.

enrichment scores and correlation analysis between the mitochondrial autophagy pathway and Glyco-DEGs are detailed in Tables SV-SVII. Western blot analysis confirmed PINK1 overexpression upregulated PGK2, showing that in both MCF-7 and MDA-MB-231 cells, PINK1 overexpression increased PGK2 protein levels, while PINK1 knockdown decreased PGK2 expression (Fig. 10H), with statistically significant differences.

Analysis identified differentially expressed glycolysis-related genes in breast cancer and correlation analysis revealed a positive association between PINK1-related mitophagy and glycolysis pathways. Western blot experiments confirmed that PINK1 overexpression increased PGK2 protein levels, whereas PINK1 knockdown decreased PGK2 expression in both MCF-7 and MDA-MB-231 cells.

PINK1-driven PGK2 activation enhances glucose uptake. Building on previous evidence that PINK1 may promote glycolysis by upregulating PGK2, it was investigated whether this metabolic shift enhances glucose uptake in breast cancer cells. In total, two siRNAs targeting PGK2 were designed. siPGK2-2 showed the best knockdown efficiency and was selected for subsequent experiments (Fig. S4). Glucose uptake was quantified using a fluorescent glucose analog and flow cytometry under three experimental conditions in both MCF-7 and MDA-MB-231 cells: Control vector, PINK1-OE and PINK1-OE + siPGK2. As illustrated in Fig. 11A, PINK1 overexpression resulted in a significant increase in glucose-positive cell populations, rising from 53.53 to 71.57% in MCF-7 cells and from 73.21 to 94.67% in MDA-MB-231 cells. This

elevation was markedly reduced when PGK2 was knocked down, as shown by quantitative analyses (Fig. 11B and C).

These results suggest a potential role for PINK1 in promoting glycolytic activation in breast cancer cells, possibly involving PGK2, although direct mechanistic evidence is lacking. By enhancing glucose utilization, PINK1 facilitates metabolic reprogramming that supports the high energy demands of rapidly proliferating and migrating cancer cells. These results indicate that PINK1 modulates PGK2 expression in breast cancer cells.

PGK2-dependent migration assay following PINK1 overexpression. Given the prior findings that PINK1 may enhance glycolysis and glucose uptake through PGK2 activation, it was next examined whether this metabolic adaptation contributes to the migratory behavior of breast cancer cells. Wound-healing assays were performed using MCF-7 and MDA-MB-231 cells under three conditions: Control vector, PINK1-OE and PINK1-OE + siPGK2. As depicted in Fig. 12A and B, PINK1-OE significantly accelerated wound closure after 24 h in both cell lines, indicative of enhanced cell migration. Quantitative analysis substantiated a significant increase in relative migration distance compared with the control group (Fig. 12C and D). However, co-transfection with siPGK2 notably diminished this migratory enhancement, suggesting a critical role for PGK2 in mediating PINK1-induced cell migration. In MCF-7 cells, PINK1 overexpression resulted in an ~80% increase in migration compared with the control group, while co-transfection with siPGK2 mitigated this enhancement by ~28%. A similar trend was observed in

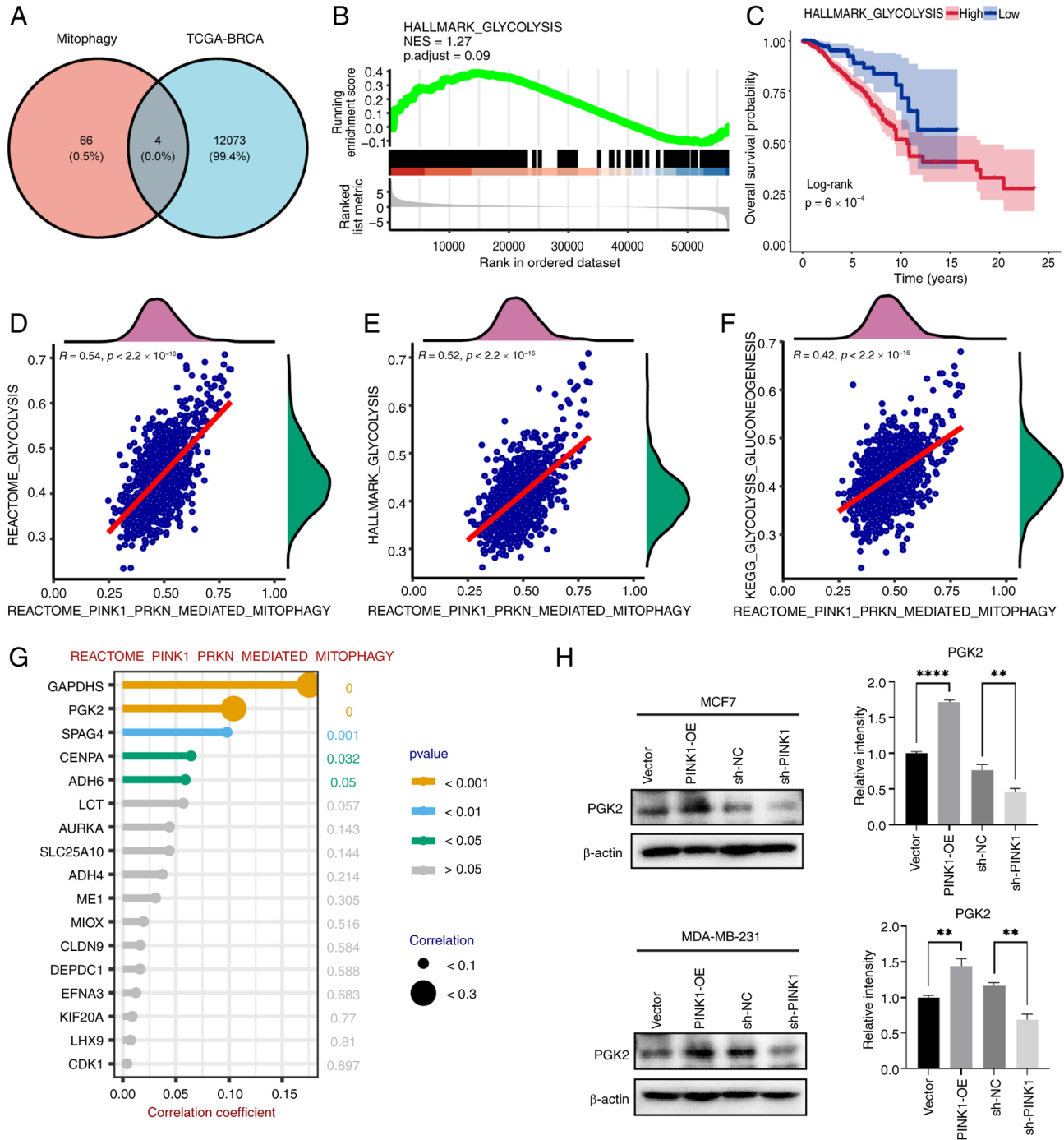


Figure 10. Correlation analysis of PINK1-mediated mitophagy with glycolysis and patient prognosis in breast cancer. (A) Intersection analysis between DEGs and Glyco-DEGs in the TCGA-BRCA dataset. (B) GSEA analysis of the glycolysis pathway in the TCGA-BRCA dataset. (C) Prognostic analysis of the glycolysis pathway activity in the TCGA-BRCA cohort. (D) Correlation between the glycolysis score and the activity of the REACTOME_PINK1_PRKN_MEDIATED_MITOPHAGY pathway. (E) Correlation between the PINK1-mediated mitophagy pathway activity and hallmark glycolysis gene set activity. (F) Correlation between the PINK1-mediated mitophagy pathway activity and glycolysis core signature gene set activity. (G) Correlation analysis between the PINK1-mediated mitophagy pathway and glycolysis differential genes. (H) Western blot analysis of PGK2 protein expression in MCF-7 and MDA-MB-231 cells following PINK1 overexpression or knockdown. Quantification of PGK2 levels is shown on the right, normalized to β -actin. Statistical analyses were performed using GSEA, log-rank test and Spearman correlation for the bioinformatics data, and unpaired two-tailed Student's t-test for experimental validation. Data represent means $\pm$ SEM from at least three independent experiments. ** $P < 0.01$, **** $P < 0.0001$. PINK1, PTEN-induced kinase 1; DEGs, differentially expressed genes; Glyco, glycolysis; TCGA, The Cancer Genome Atlas; BRCA, Breast Invasive Carcinoma; GSEA, Gene Set Enrichment Analysis; NES, Normalized Enrichment Score; PGK2, Phosphoglycerate Kinase 2.

MDA-MB-231 cells, where PINK1-induced migration was suppressed following PGK2 inhibition. Collectively, these data provide functional evidence that PINK1 may promote breast cancer cell migration via a PGK2-dependent metabolic

mechanism. Unlike prior studies that primarily focused on glucose uptake, this experiment established a link between PINK1-driven glycolysis and enhanced motility (56,57). These findings support a broader model wherein PINK1, through

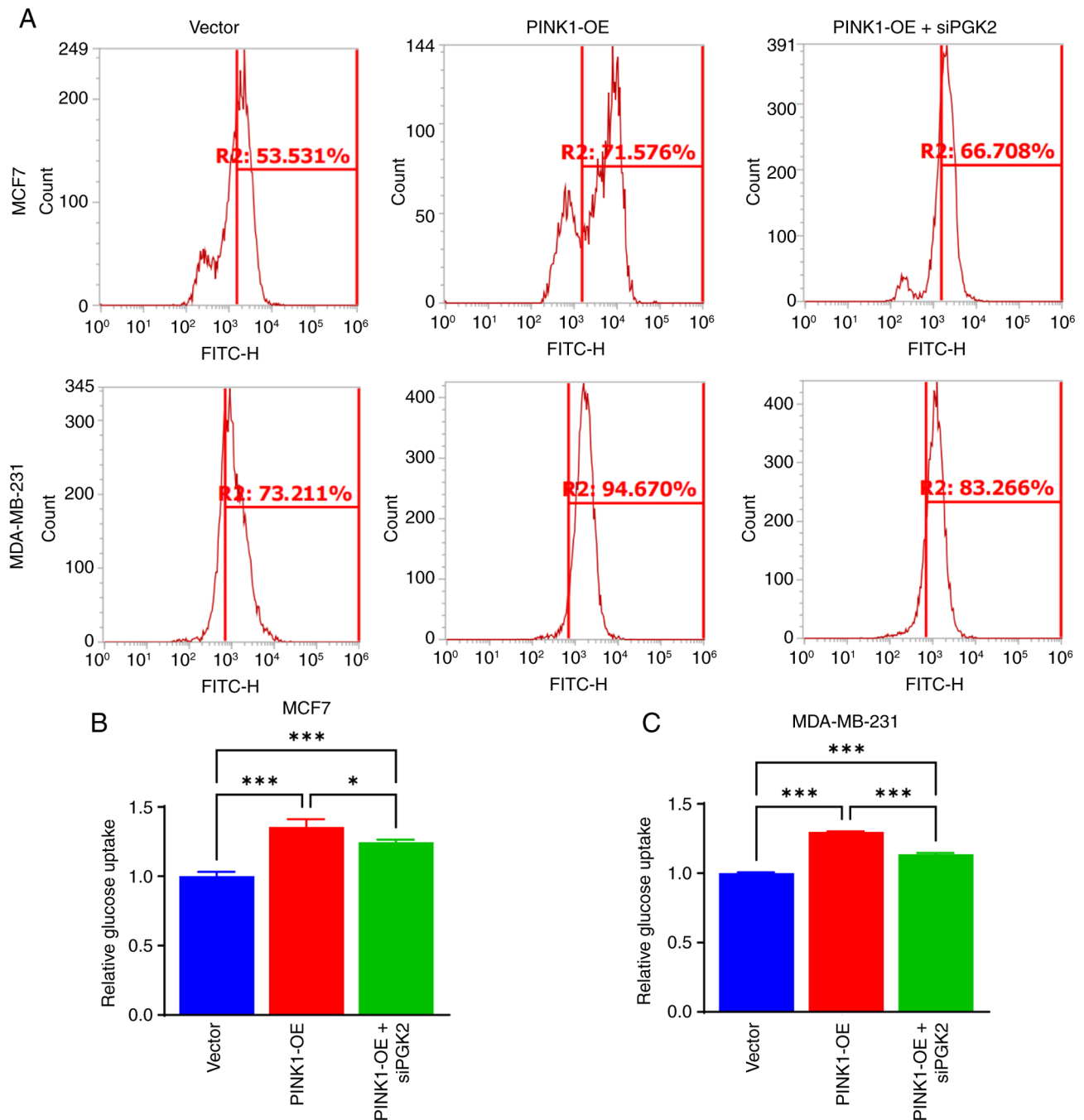


Figure 11. Flow cytometric analysis of glucose uptake in breast cancer cells upon PINK1 overexpression and PGK2 knockdown. (A) Flow cytometric analysis of glucose uptake in MCF-7 and MDA-MB-231 cells transfected with vector control, PINK1-OE or PINK1-OE + siPGK2. Quantitative comparison of glucose-positive cell populations in (B) MCF-7 and (C) MDA-MB-231 cells. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. Data are represented as the mean $\pm$ SEM from at least three independent experiments. * $P < 0.05$, *** $P < 0.001$. PINK1, PTEN-induced kinase 1; OE, overexpression; si, small interfering; PGK2, phosphoglycerate kinase 2.

mitophagy-induced metabolic reprogramming, facilitates both energy production and the acquisition of aggressive phenotypes that drive breast cancer progression.

Glycolytic activation by PINK1 promotes invasion. To further elucidate the link between PINK1-induced metabolic rewiring and invasive potential, Transwell invasion assays were conducted in both MCF-7 and MDA-MB-231 breast cancer cells under three experimental conditions: Vector control, PINK1-OE and PINK1-OE + siPGK2. As shown in Fig. 13,

PINK1 overexpression significantly enhanced the invasive capacity of both MCF-7 and MDA-MB-231 cells compared with the vector group. Quantitative analysis demonstrated a significant increase in the number of invasive cells upon PINK1-OE, confirming that PINK1 promotes cell invasion. Notably, co-transfection with siPGK2 markedly reduced this invasive phenotype, indicating that PGK2 may be essential for PINK1-mediated invasion. In MCF-7 cells, PINK1-OE nearly doubled the number of invasive cells compared with the control, while PGK2 knock down partially reversed this effect.

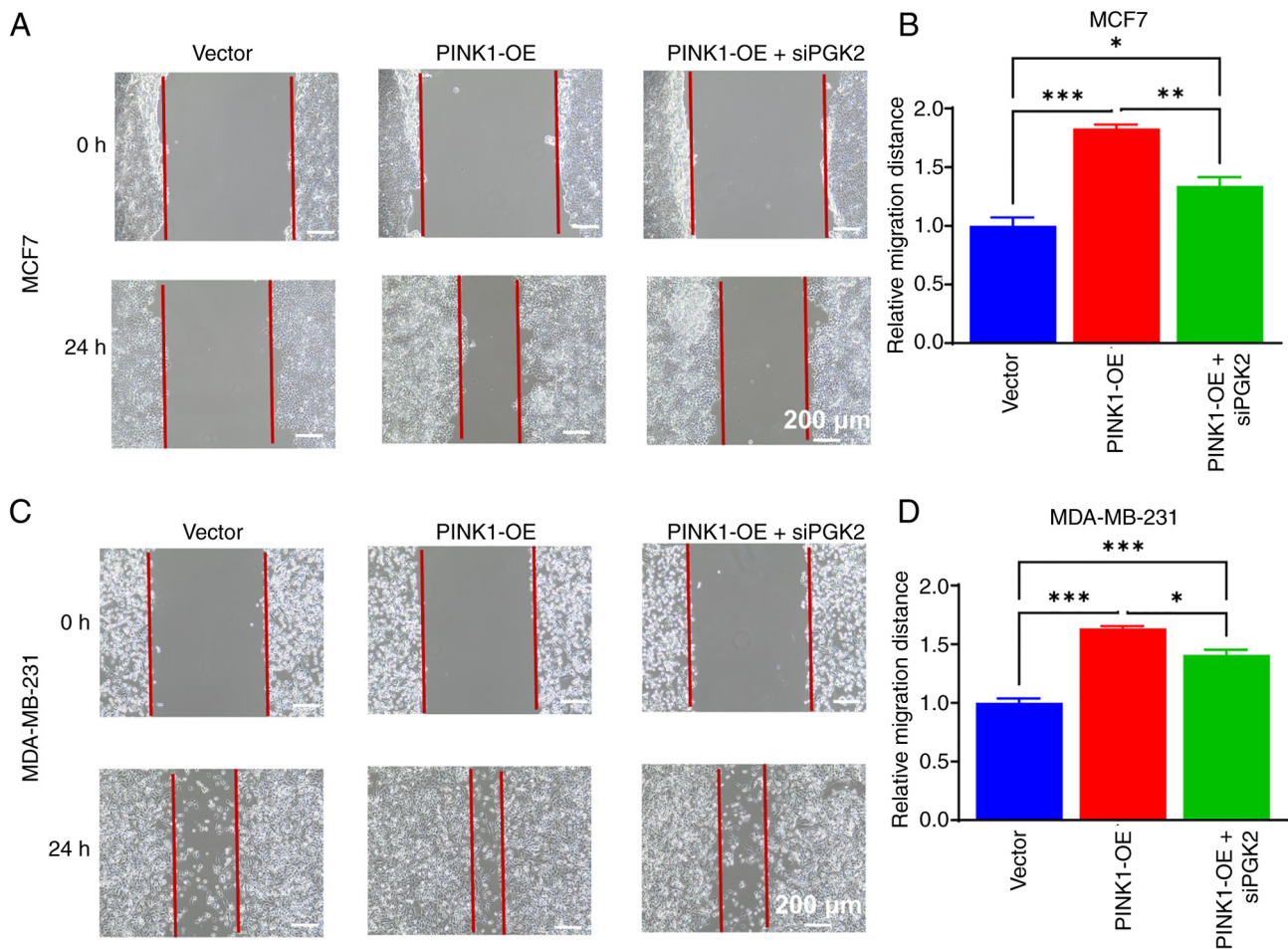


Figure 12. Wound healing assay evaluating the effect of PINK1 overexpression and PGK2 knockdown on cell migration in breast cancer cells. (A) Representative images of wound healing in MCF-7 cells transfected with vector control, PINK1-OE or PINK1-OE + siPGK2 groups at 0 and 24 h. (B) Quantitative analysis of the relative migration distance in MCF-7 cells. (C) Representative images of wound healing in MDA-MB-231 cells transfected with vector control, PINK1-OE or PINK1-OE + siPGK2 groups at 0 and 24 h. (D) Quantitative analysis of the relative migration distance in MDA-MB-231 cells. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. Data are represented as the mean $\pm$ SEM from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. PINK1, PTEN-induced kinase 1; OE, overexpression; si, small interfering; PGK2, phosphoglycerate kinase 2.

A similar trend was observed in MDA-MB-231 cells, where PGK2 knockdown diminished PINK1-induced invasion.

These findings provide functional evidence that PINK1 may promote breast cancer invasiveness through PGK2-dependent glycolytic activation. By linking mitophagy and metabolic reprogramming, the PINK1-PGK2 axis supplies the energy and biosynthetic capacity required for tumor invasion and metastasis. This underscores the potential of targeting PINK1-mediated metabolic pathways as a therapeutic approach for aggressive breast cancer subtypes.

PINK1-PGK2 axis elevates the levels of glycolytic metabolites. Given the possible role of PINK1 in driving glycolysis and invasion via PGK2, it was next examined how this regulatory axis may influence key metabolic intermediates, particularly acetyl-CoA. Acetyl-CoA is central to metabolism, linking glycolysis to the tricarboxylic acid (TCA) cycle while serving as a critical molecule for energy production and biosynthesis; it fuels the TCA cycle for ATP generation and supports processes such as fatty acid synthesis and protein acetylation, which affect gene expression and cellular growth.

Utilizing ELISA assays, the PGK2, pyruvate and acetyl-CoA levels in MCF-7 and MDA-MB-231 breast cancer cells were quantified under three conditions: Vector control, PINK1-OE and PINK1-OE + siPGK2. In MCF-7 cells (Fig. 14A-C), PINK1-OE significantly increased PGK2 expression and elevated the levels of pyruvate and acetyl-CoA, indicating enhanced glycolytic flux and metabolic activation. Co-transfection with siPGK2 partially reduced these elevations, confirming the dependence of this effect on PGK2 activity. Consistent results were observed in MDA-MB-231 cells (Fig. 14D-F), where PINK1-OE upregulated PGK2 and its downstream metabolites, all of which decreased upon siPGK2 treatment. These results demonstrate that PINK1 upregulation enhances glycolytic throughput, leading to the accumulation of pyruvate and acetyl-CoA, key intermediates that link cytosolic glycolysis to mitochondrial energy metabolism. Elevated acetyl-CoA reflects an increased capacity for TCA cycle activity and biosynthetic processes, supporting tumor cell growth and metabolic flexibility. Furthermore, PGK2 knockdown partially suppressed acetyl-CoA accumulation, confirming the dependence of this metabolic shift on glycolysis. These findings underscore the PINK1-PGK2 axis as

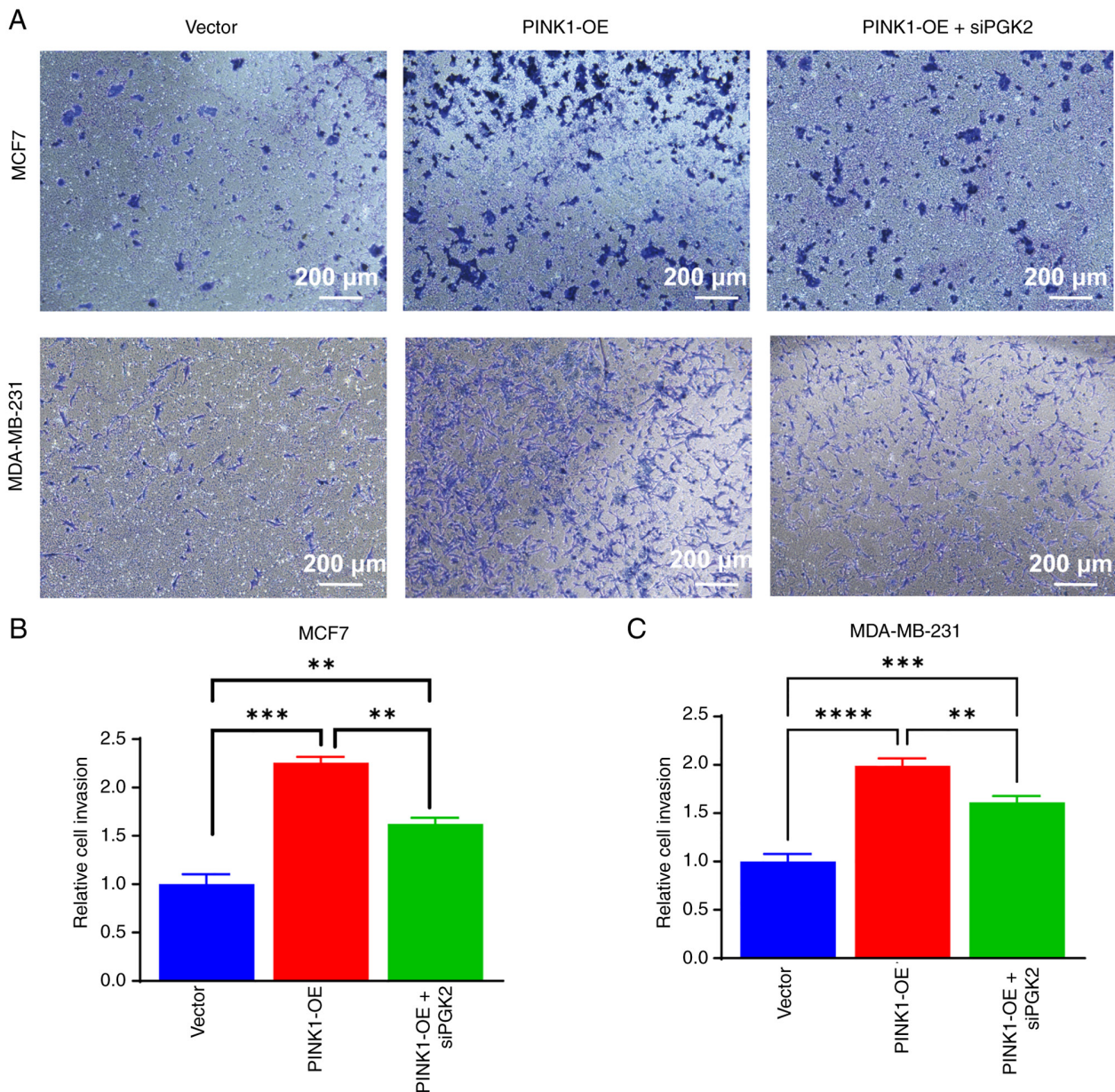


Figure 13. Mechanistic analysis of the PINK1-PGK2 axis in regulating breast cancer invasion through metabolic reprogramming. (A) Representative images of Transwell invasion assays with MCF-7 cells and MDA-MB-231 cells transfected with vector control, PINK1-OE or PINK1-OE + siPGK2. Quantitative analysis of invasive cell numbers under the indicated conditions in MCF-7 (B) cells and MDA-MB-231 (C) cells. Statistical analysis was performed using a one-way ANOVA followed by Tukey's post-hoc test. Data are represented as the mean $\pm$ SEM from at least three independent experiments. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. PINK1, PTEN-induced kinase 1; OE, overexpression; si, small interfering; PGK2, phosphoglycerate kinase 2.

a potentially pivotal regulator of glycolysis and a link between glycolytic reprogramming and mitochondrial function.

Overall, the results of the present study indicate that the PINK1-PGK2 axis may play a central role in metabolic reprogramming in breast cancer cells, enhancing glycolysis, mitochondrial function and biosynthesis (Fig. S5). This metabolic flexibility aids tumor cells in survival and proliferation, rendering this axis a promising target for future cancer therapies.

Discussion

Breast cancer remains a leading cause of cancer-related mortality, primarily due to its aggressive nature and the

limited availability of effective targeted therapies (58). A hallmark of cancer progression is metabolic reprogramming, characterized by a shift towards glycolysis (59). However, the mechanisms linking mitochondrial quality control to metabolism in TNBC are not yet fully understood (60). A prior study has demonstrated that PINK1, a critical regulator of mitophagy, is vital for maintaining mitochondrial integrity (61). However, the specific role of PINK1 in connecting mitophagy to glycolytic reprogramming in breast cancer has not, to the best of our knowledge, been extensively investigated. Elevated PINK1 expression in TNBC correlates with poor prognosis, suggesting its potential role in tumor progression (62). Recent findings indicate that PINK1 may influence glycolytic flux through enzymes such as PGK2 (63). PGK2,

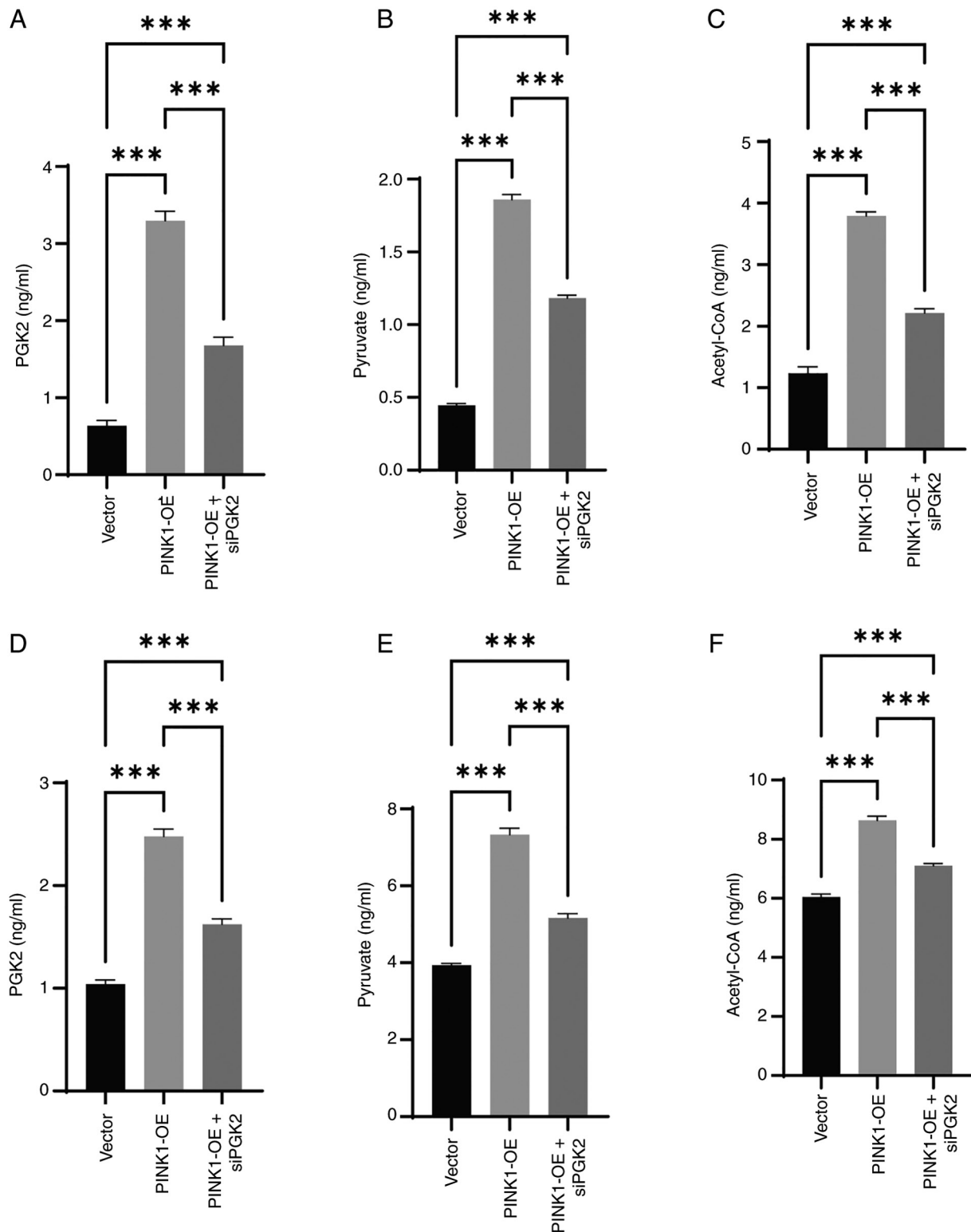


Figure 14. Analysis of glycolysis-associated metabolite level changes in MCF-7 and MDA-MB-231 breast cancer cells. Levels of (A) PGK2, (B) pyruvate and (C) acetyl-CoA in MCF-7 cells, and (D) PGK2, (E) pyruvate and (F) acetyl-CoA in MDA-MB-231 cells transfected with vector control, PINK1-OE or PINK1-OE + siPGK2. Quantitative analysis was performed using ELISA in cells under the three indicated conditions. Statistical analysis was performed using a one-way ANOVA followed by Tukey's post-hoc test. Data are represented as the mean $\pm$ SEM from at least three independent experiments. *** $P < 0.001$. PGK2, phosphoglycerate kinase 2; PINK1, PTEN-induced kinase 1; OE, overexpression; si, small interfering.

an important enzyme in the glycolytic pathway, catalyzes the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate, thereby facilitating ATP production (64,65). While glycolysis is recognized as a central component of tumor metabolism,

the exact relationship between PINK1-mediated mitophagy and glycolytic reprogramming in breast cancer cells has yet to be thoroughly explored. The present study aimed to address this gap by elucidating the PINK1-PGK2 axis as a

potential fundamental mechanism linking mitochondrial turnover to glycolytic reprogramming, thereby promoting aggressive tumor growth and treatment resistance in TNBC. Targeting this PINK1-PGK2 axis presents a promising therapeutic strategy for exploiting the metabolic vulnerabilities of aggressive breast cancer. By inhibiting this pathway, it may be possible to disrupt the metabolic flexibility that this cancer type utilizes for survival and progression.

The findings of the present study provide compelling evidence that PINK1 may play a crucial oncogenic role in breast cancer by integrating mitochondrial quality control with metabolic reprogramming, which is essential for meeting the energy demands and biosynthetic processes necessary for tumor proliferation and invasion, particularly in aggressive subtypes such as TNBC. Specifically, the overexpression of PINK1 in both luminal and TNBC models significantly activated the mitophagy pathway, as evidenced by elevated levels of Parkin and LC3-II and an increase in autophagosomal structures observed through TEM. This activation of mitophagy was not a mere passive response but actively facilitated cellular proliferation, migration and invasion, thereby reinforcing the oncogenic adaptation mediated by PINK1.

Furthermore, the present study identified PGK2 as a glycolytic effector downstream of PINK1. The mechanistic data indicated that PINK1 overexpression upregulated PGK2, resulting in enhanced glycolytic flux, increased glucose uptake and the accumulation of critical metabolites such as pyruvate and acetyl-CoA. Notably, acetyl-CoA is a central metabolite linking glycolysis to the TCA cycle and other anabolic pathways; its increased availability suggests that PINK1 not only augments glycolysis but also enhances pyruvate dehydrogenase activity, facilitating the flow of carbon into the TCA cycle (66). This process supports mitochondrial ATP production and generates citrate for lipid biosynthesis via ATP citrate lyase. The resulting cytosolic acetyl-CoA fuels *de novo* fatty acid synthesis through enzymes such as acetyl-CoA carboxylase and fatty acid synthase. This process supports membrane biogenesis in rapidly proliferating cells (67). Therefore, the PINK1-PGK2 axis may orchestrate a complex metabolic shift that meets energetic and biosynthetic demands. We hypothesize that this axis also interacts with other regulatory pathways, such as enhanced histone acetyltransferase activity and lipogenesis, to establish a metabolically aggressive and transcriptionally permissive tumor phenotype. This hypothesis is primarily based on the present finding that the PINK1-PGK2 axis significantly elevated the levels of acetyl-CoA, a key precursor for both epigenetic modifications and lipid synthesis. Consistently, pharmacological inhibition of PGK2 reversed a number of these oncogenic effects, thereby confirming that the PINK1-mediated program is critically dependent on glycolytic flux and the subsequent availability of these metabolic intermediates. These findings suggest that mitophagy is not merely a passive degradation process; rather, it actively contributes to biosynthetic and energetic adaptation in breast cancer cells, fulfilling the metabolic requirements for rapid tumor progression.

In addition to metabolic flux, PINK1-driven mitophagy may influence broader transcriptional environments associated with glycolytic regulation. First, increased glycolytic demand and shifts in oxygen/redox states can stabilize

hypoxia-inducible factor 1α , a master regulator of glycolytic gene expression, which could indirectly favor PGK2 upregulation (68,69). Second, p53, a key metabolic gatekeeper that constrains glycolysis (70), has been reported to intersect with PINK1-associated stress responses, raising the possibility that p53 loss or inhibition may further facilitate PGK2 induction downstream of PINK1 (66). Based on the results of the present study, we propose the following testable hypotheses: i) PINK1 positively regulates PGK2 expression, placing PGK2 downstream of PINK1 signaling; and ii) restoration of wild-type p53 activity attenuates PINK1-induced upregulation of PGK2. To interrogate the functional role of PGK2 in PINK1-mediated metabolic reprogramming, genetic knockdown of PGK2 provides a specific and reliable experimental approach. Unlike pharmacologic inhibitors, which may have off-target effects, PGK2 knockdown provides a more precise model for studying the direct involvement of PGK2 in metabolic pathways. The genetic knock down of PGK2 eliminates potential confounding factors, ensuring that observed changes in pyruvate and acetyl-CoA levels are directly attributable to PGK2 activity. This approach strengthens the causal link between PGK2 and metabolic adaptation, offering a clearer understanding of the role of PGK2 in cellular metabolism under stress conditions. Future studies should explore whether additional transcriptional regulators, such as c-Myc and NF- κ B, cooperate to fine-tune PGK2 expression in a subtype-specific manner.

PINK1-mediated glycolytic reprogramming not only enhances cellular proliferation but also promotes aggressive phenotypes, such as increased migration and invasiveness. This was demonstrated in the present study through wound healing and Transwell invasion assays, where cells overexpressing PINK1 exhibited significantly greater migratory and invasive capabilities. Notably, these effects were diminished by PGK2 knockdown, reinforcing the notion that PINK1 may drive metabolic alterations that heighten cancer cell aggressiveness. Collectively, these findings establish the PINK1-PGK2 axis as a potential link between mitochondrial turnover and the metabolic flexibility essential for tumor invasiveness.

Supporting the clinical relevance of the findings of the present study, data mining from TCGA-BRCA cohorts revealed a robust association between elevated PINK1 expression and poor patient outcomes, particularly in TNBC subtypes. Future research should investigate whether targeting PINK1 can modify the immune microenvironment and enhance the efficacy of immunotherapy. Beyond metabolic control, the PINK1-PGK2 axis may also influence immune contexture in breast cancer. Recent single-cell studies of IL27RA and TMEM71 demonstrated a coordinated reprogramming of metabolic and immune pathways in breast tumors, linking metabolic alterations to immune modulation and disease progression (71,72). These data align with the observation in the present study that PINK1-driven glycolytic activation may be associated with immunosuppressive features and adverse outcomes. Furthermore, systemic immune-inflammatory and nutritional indices independently predict prognosis in breast cancer, underscoring the systemic nature of tumor-host metabolic interactions (73). Complementary Mendelian randomization further supports a causal link between metabolic changes and immune-related diseases (74). Collectively, these studies

suggest a potential link between PINK1-related metabolic regulation and immune modulation, implying that the PINK1-PGK2 axis may connect metabolic function with immune responses. This positioning suggests that inhibition of this glycolytic pathway could both restrict tumor growth and recalibrate antitumor immunity. We propose testing metabolic-immunotherapy combinations in TNBC, guided by PINK1/PGK2 expression and immune-nutritional indices, to refine patient selection and elucidate mechanistic connections. The results of the present study indicate that PINK1 may contribute to metabolic reprogramming and immune evasion, complicating the prognosis and treatment of TNBC.

In conclusion, the present study identified PINK1 as a potential regulator of breast cancer progression by elucidating the connection between mitochondrial quality control and metabolic reprogramming. It was demonstrated that PINK1-mediated mitophagy upregulated the downstream glycolytic enzyme PGK2. This upregulation enhanced glucose uptake, pyruvate generation and acetyl-CoA accumulation. These metabolic alterations facilitated proliferation, migration and invasion in both luminal and TNBC cells. Knocking down PINK1 impaired mitophagy, suppressed glycolysis and diminished malignant characteristics. Mechanistically, the PINK1-PGK2 axis emerged as a possible metabolic bridge that promotes energy flow and biosynthetic activity. Additionally, increased PINK1 expression correlated with immunosuppressive microenvironments and adverse patient prognosis. Functionally, knocking down PGK2 effectively counteracted PINK1-induced tumor progression, underscoring the therapeutic significance of this pathway. The findings highlighted the dual oncogenic roles of PINK1 in maintaining mitochondrial integrity and driving glycolytic adaptation. Targeting the PINK1-PGK2 metabolic axis may offer promising strategies for addressing aggressive, metabolically flexible and treatment-resistant breast cancer subtypes. Furthermore, combining inhibitors of the PINK1-PGK2 axis with existing chemotherapies or metabolic modulators could yield synergistic effects and overcome drug resistance. Overall, the present study provided mechanistic insights into the intersection of mitophagy and metabolic reprogramming in breast cancer; it established the PINK1-PGK2 axis as a promising target for therapeutic intervention, particularly in metabolically adaptable and treatment-resistant subtypes such as TNBC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZG contributed to study design, formal analysis and writing the original draft. QY contributed to conceptualization, project administration and reviewing and editing the manuscript. RS contributed to data collection, preprocessing data and writing the original draft. WL contributed to the statistical analysis of the present study. HD contributed to conceptualization, supervision, funding acquisition and reviewing and editing the manuscript. WL and HD confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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