Article

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NPM1 Serves as a Tumor-Promoting Gene in Lung Adenocarcinoma by Regulating Glycolysis

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Background: The role of Recombinant Nucleophosmin 1 (*NPM1*) in the development of lung adenocarcinoma (LUAD) has been recognized, however, the precise mechanism underlying this involvement remains unclear. Therefore, this study aimed to assess the mechanism of *NPM1* in LUAD progression.

Methods: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting assays were used to detect the expression level of *NPM1*. Furthermore, Cell Counting Kit-8 (CCK-8) assay, Transwell migration, Transwell invasion, and flow cytometry assays were conducted to evaluate the role of NPM1 in the proliferation, migration, invasion, and apoptosis in LUAD cell lines. Moreover, glycolysis-related indicators were determined using corresponding kits. Additionally, the mechanism of *NPM1* involvement was further evaluated through *NPM1* silencing experiments.

Results: *NPM1* was upregulated in two human LUAD cell lines ($p < 0.001$). Glycolysis was enhanced in LUAD cell lines ($p < 0.05$). After inhibiting the expression of *NPM1*, the proliferation, migration, and invasion were significantly reduced in LUAD cell lines but the apoptosis was increased ($p < 0.05$). Furthermore, a substantial reduction in glycolytic flux and an elevation in mitochondrial respiration were observed ($p < 0.05$).

Conclusion: These findings reveal that *NPM1* promotes LUAD progression by mediating glycolysis, offering a novel therapeutic target for treating LUAD.

Keywords: lung adenocarcinoma; *NPM1*; LUAD development; glycolysis

Introduction

Lung cancer accounts for 25% of cancer-related deaths, with the highest incidence among men aged ≥40 and women aged ≥60 [1]. Lung adenocarcinoma (LUAD) is a prominent subtype of lung cancer [2]. Over the past few years, there has been a significant increase in LUAD morbidity, making it one of the most invasive types of lung cancer [3]. Despite considerable advancement in LUAD treatment, the incidence and death rates continue to rise, posing a serious threat to human health. In recent years, there has been increasing interest in reprogramming energy metabolism in studying LUAD progression and treatment [4].

The reprogramming of energy metabolism is defined by a phenomenon known as the "Warburg effect", generally acknowledged as a hallmark of cancers [5]. Tumor cells prefer to reprogram a myriad of glucose metabolism reactions towards glycolysis, even in oxygen-rich environments [6]. This glycolytic process contains increased glucose uptake and lactate production alongside decreased adenosine triphosphate (ATP) production [7]. Based on this finding, researchers have delved into the metabolic landscape of lung cancer, indicating a disruption in glucose metabolism [8,9]. The molecular mechanism underlying disturbed energy metabolism in LUAD is widely investigated. For example, LncRNA FAM83A-AS1 and LINC01123 have been reported to promote LUAD cell proliferation and migration by enhancing glycolysis [10,11]. Additionally, Fascin and BACH1 have been identified to facilitate lung cancer metastasis by improving glycolysis [12,13]. Hence, development of metabolic interventions targeting glycolysis could be a promising approach for LUAD treatment.

In recent years, the role of Recombinant Nucleophosmin 1 (*NPM1*) in solid tumors has been increasingly studied. *NPM1*, a multifunctional protein mainly located in the nucleolus with shuttling between the nucleus and cytoplasm, has emerged as a marker for a poor prognosis of leukemia [14]. Researchers have uncovered that *NPM1* is overexpressed in various cancer types and promotes tumor development [15,16]. As a result, anti-tumor drugs targeting *NPM1* have been extensively studied [17]. In LUAD, overexpression of *NPM1* is closely linked with cancer cell glycolysis, although the specific mechanism remains unknown [18]. This study highlights the significance of elucidating the relevant mechanism by which *NPM1* functions in LUAD, aiming at improving the efficacy of targeted treatments for LUAD patients.

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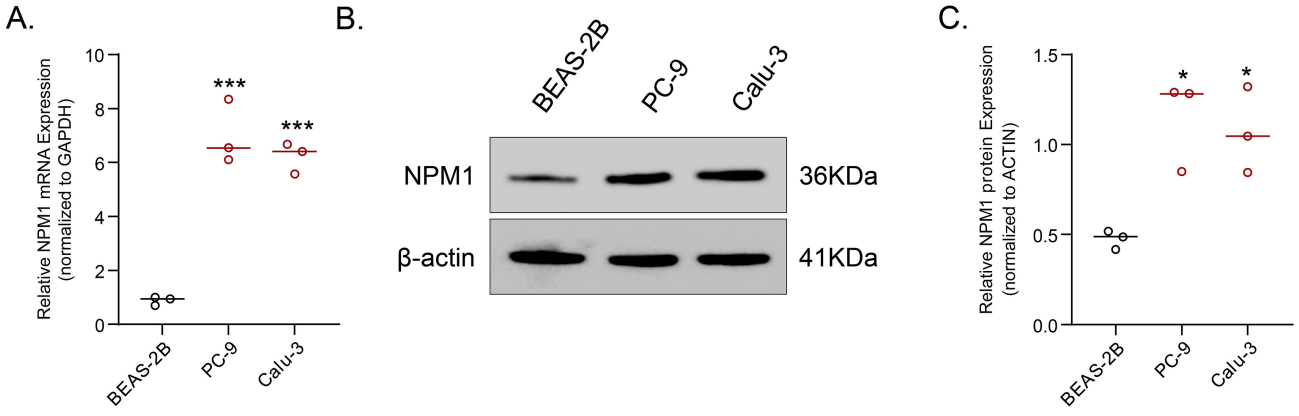

Fig. 1. The expression levels of Recombinant Nucleophosmin 1 (*NPM1*) in lung adenocarcinoma (LUAD) cell lines. (A) The expression levels of *NPM1* in the normal human bronchial epithelial cells BEAS-2B and two human lung cancer cell lines (PC-9 and Calu-3) were assessed using RT-qPCR assay. (B,C) *NPM1* expression in lung cancer cell lines was evaluated using western blot analysis. $*p < 0.05$, $***p < 0.001$ compared to the BEAS-2B. n = 3. RT-qPCR, reverse transcription-quantitative polymerase chain reaction, BEAS-2B, human bronchial epithelial cells.

In this study, we validated the expression of *NPM1* and evaluated the glycolysis status in LUAD samples or cell lines. Additionally, we investigated the intrinsic mechanism between *NPM1* and aberrant glycolysis. Our findings revealed how *NPM1* promotes LUAD development by modulating glycolysis. Furthermore, this study confirmed that targeting *NPM1* can potentially improve the efficacy of LUAD therapy.

Materials and Methods

Cell Culture

The human LUAD cell lines PC-9 (BNCC340767), Calu-3 (BNCC359757), and healthy lung cell lines human bronchial epithelial cells (BEAS-2B) (BNCC359274) were obtained from the BeNa Culture Collection (BNCC, Beijing, China). The cell lines were authenticated using STR profiling and tested negative for cross-contamination utilizing the mycoplasma testing. The cells were cultured in RPMI-1640 (cat no. 11875093, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (cat no. A5669401, Gibco, Grand Island, NY, USA) and incubated in an atmosphere of 5% CO_2 at 37 °C.

Cell Transfection

The *NPM1* pcDNA3.1 vector (*NPM1*), along with negative control empty vectors pcDNA3.1 (NC), as well as *NPM1* siRNAs (si-*NPM1*#1, si-*NPM1*#2, and si-*NPM1*#3) and si-NC were designed and synthesized by Invitrogen (Carlsbad, CA, USA). The cells were transfected employing Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Following 48 hours of transfection, the cells were harvested and underwent transfection efficiency assay and subsequent experiments. Vector sequences used in transfection experiments were as follows: si-*NPM1*#1: 5′-CACCAGTGGTCTTAAGGTTGA-3′; si-*NPM1*#2: 5′-AAGGACAAG AATCCTTCAAGA-3′; si-*NPM1*#3: 5′-CCUAGUUCUGUAGAAGACA-3′; si-*NC*: 5′-UUCUUCGAACGUGUCACGUTT-3′.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRIzol Reagent (cat no.15596026CN, Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized utilizing an All-in-One First Strand cDNA synthesis kit (cat no.11119ES60, YEASEN, Shanghai, China). Furthermore, qPCR SYBR Green Master Mix (cat no. 11201ES50, YEASEN, Shanghai, China) was used to perform qRT-PCR. Relative expression of the target genes was assessed utilizing the $2^{-\Delta\Delta Ct}$ method. *GAPDH* was used as an internal control in this assay.

The primers used in amplification were as follows: *NPM1* forward, 5′-TCCTGGAGGTGGTAACAAGG-3′, *NPM1* reverse, 5′-ACCCTTTGATCTCGGTGTTG-3′. *GAPDH* forward, 5′-GCACCGTCAAGGCTGAGAAC-3′, *GAPDH* reverse, 5′-TGGTGAAGACGCCAGTGGA-3′.

Western Blotting (WB) Assay

Total protein was extracted using RIPA buffer (cat no. P0013B, Beyotime Institute of Biotechnology, Shanghai, China), followed by separation of the proteins on 10% SDS-PAGE. Subsequently, the proteins were transferred onto PVDF membranes. The membranes were blocked with a blocking buffer at room temperature for one hour. After this, the membranes were incubated overnight with primary antibodies against *NPM1* (1:1000, ab10530, Abcam, Boston, MA, USA) and *β-actin* (1:1000, ab8226, Abcam, Boston, MA, USA). The next day, the mem-

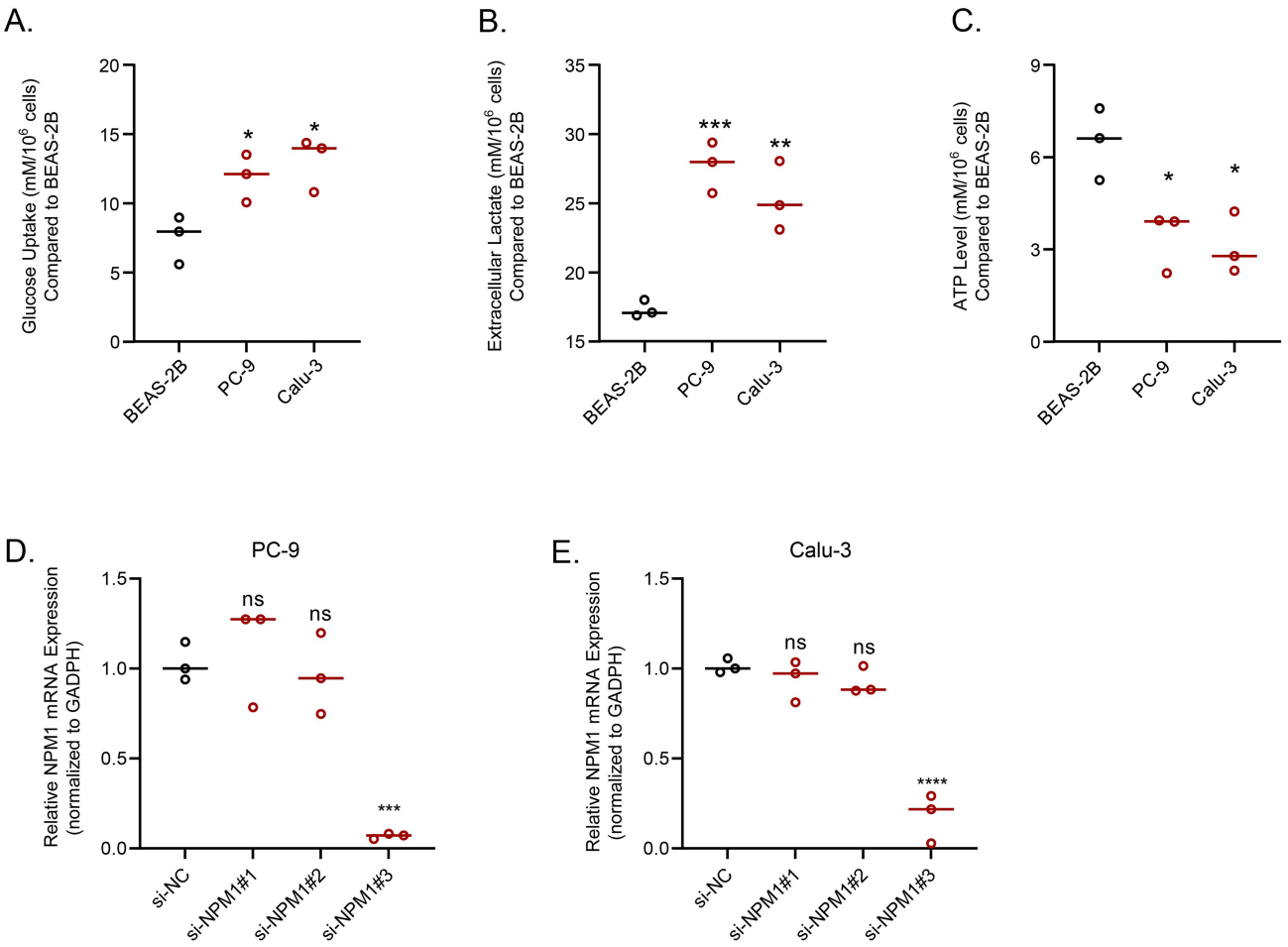

Fig. 2. Assessment of glycolytic energy metabolism in LUAD cell lines and NMP1 inhibitory vector expression. (A) Glucose uptake assay. (B) Extracellular lactate level determination. (C) Adenosine triphosphate (ATP) level determination. (D,E) Evaluation of transfection effect. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, no significance. n = 3.

branes underwent incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody IgG (1:2000, ab205719, Abcam, Boston, MA, USA). Finally, the protein bands were visualized using the chemiluminescence kit (cat no. WBAVDCH01, EMD Millipore, Billerica, MA, USA), and images were captured using a Bio-Rad ChemiDoc XRS plus Imager (cat no.1108763S, Bio-Rad, Hercules, CA, USA). Moreover, protein bands were quantified utilizing ImageJ software (version 1.8.0, LOCI at the University of Wisconsin, Madison, WI, USA).

Cell Counting Kit-8 (CCK-8) Assay

The transfected si-*NPM1* and si-NC cells were seeded into 96-well culture plates (1×10^4/well) and treated as previously described [19]. For CCK-8 assay (cat no. C0037, Beyotime Institute of Biotechnology, Shanghai, China), after 24, 48, and 72 hours of treatment, 95 µL of medium and 5µL of CCK-8 kit were added into each well and incubated at 37 °C for 2 hours. Finally, the OD value at 450 nm was determined using a microplate reader (BioTek LogPhase 600, BioTek Instruments, Inc., Winooski, VT, USA).

Transwell Migration and Invasion Assay

The transwell migration and invasion assays were performed using a transwell chamber (cat no. 353097, Corning Inc., Tokyo, Japan), following a previously described method [20]. Briefly, 4×10^4 CRC cells were cultured in a serum-free medium and seeded into the upper chamber of the transwell inserts, while the lower chamber was filled with a medium containing 20% FBS. For the invasion assay, the upper chamber was pre-coated with Matrigel (cat no. OM-1-ph, MEGAROBO Biosictech Co., Ltd., Suzhou, China). After 15 hours of incubation, the upper chambers were gently swabbed with cotton, and the migrated cells were fixed using paraformaldehyde (cat no. 30525-89-4, Sinopharm Group Co., Ltd., Shanghai, China) for 30 minutes, followed by staining with 0.1% crystal violet dye (cat no. C0121, Beyotime Institute of Biotechnology, Shanghai, China) for 20 minutes. Finally, migrated and invaded cells were counted in three randomly selected fields employing an inverted microscope (cat no. Ti2, Nikon Instruments Inc., Tokyo, Japan).

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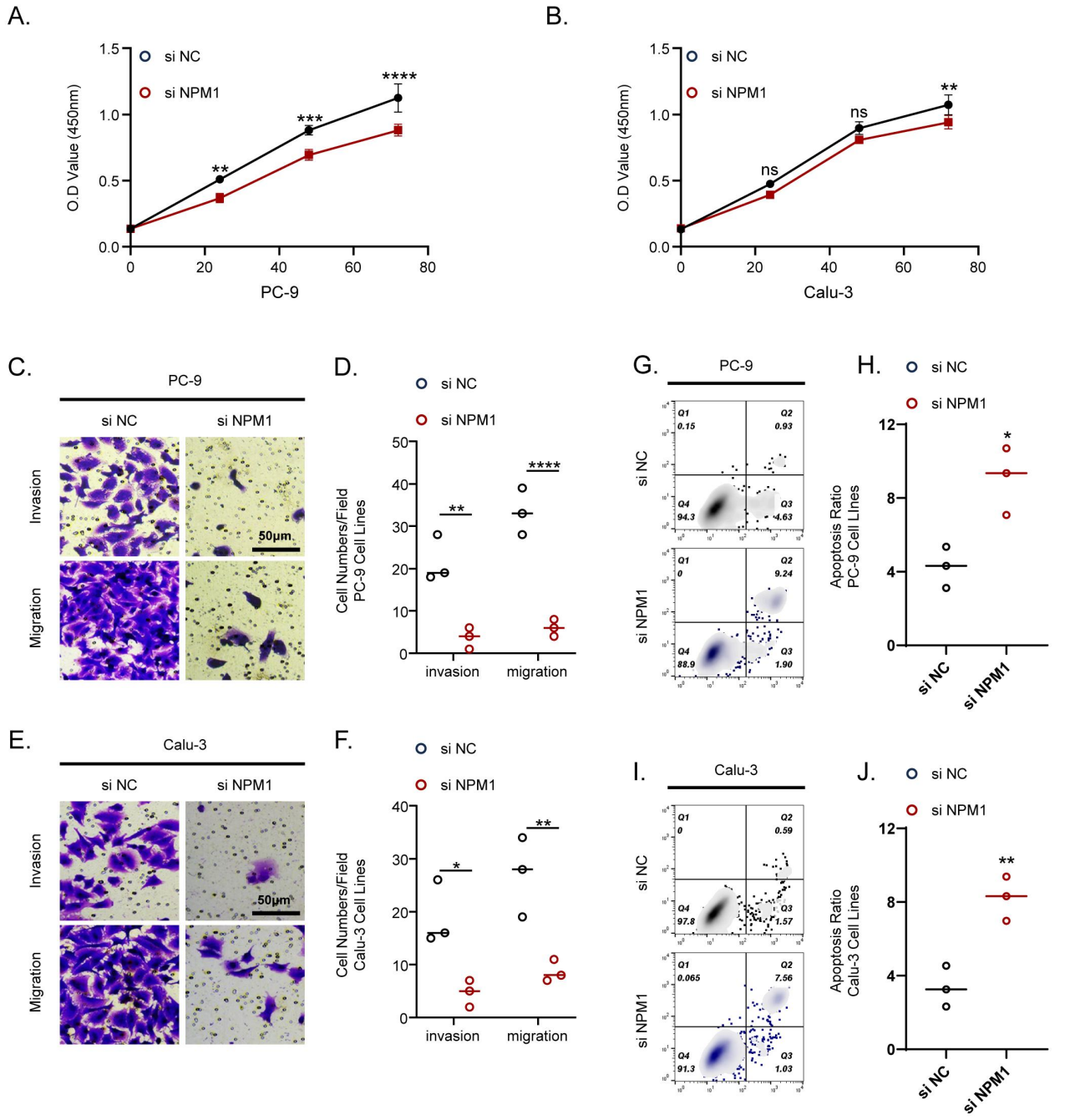

Fig. 3. *NPM1* silencing repressed NSCLC progression. (A,B) Cell Counting Kit-8 (CCK-8) assay. (C–F) Transwell migration and invasion assays. (G–J) Apoptosis assay. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, no significance, compared to the si-NC treated cell lines. n = 3.

Apoptosis Assay

PC-9 and Calu-3 cells were collected 48 hours after transfection. The transfected cells were assessed for cellular apoptosis rate using flow cytometry following a previously described method [21]. After washing, the cells were resuspended in annexin V buffer, added with 5 μL Annexin V-FITC, and incubated for 10 minutes in the dark. In the next step, 5 μL of propidium iodide (cat no. C1062L, Annexin V-FITC Detection Kit, Beyotime Biotechnology, Shanghai, China) was added and incubated for another 5 minutes. Finally, flow cytometry (BD FACSCanto II, BD Biosciences, Franklin Lake, CA, USA) was performed to evaluate cell apoptosis rate. The experiment was conducted in triplicate.

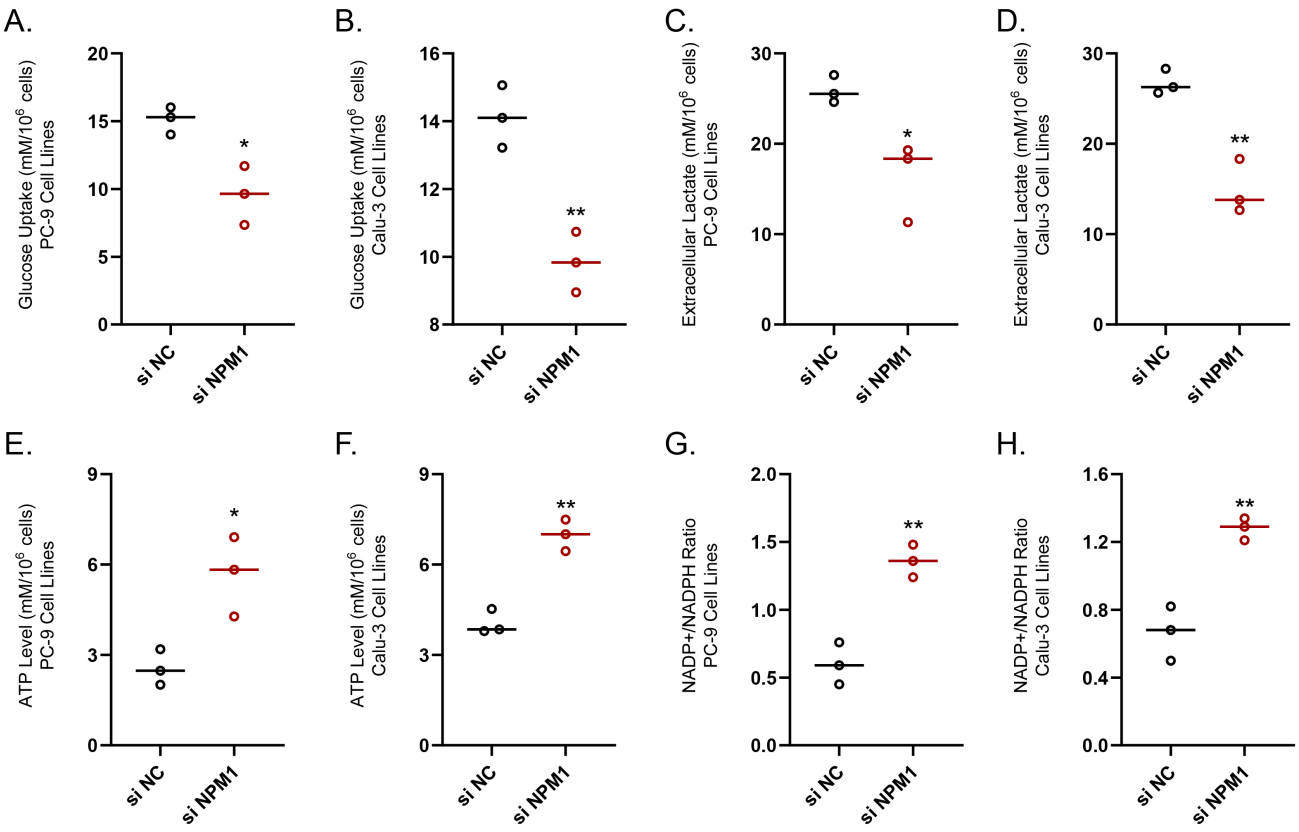

Fig. 4. The alternations in glycolytic energy metabolism following *NPM1* silencing. (A,B) Glucose uptake assay. (C,D) Extracellular lactate level determination. (E,F) ATP level determination. (G,H) NADP+/NADPH ratio determination. *$p < 0.05$, **$p < 0.01$, compared to the si-NC treated cell lines. n = 3. NADPH, nicotinamide adenine dinucleotide phosphate.

Measurements of Glucose, Lactate, and ATP Level and NADP+/NADPH Ratio

Glucose Uptake Colorimetric/Fluorometric Assay Kit (cat no. MAK263, Sigma-Aldrich, St. Louis, MO, USA), Lactate Colorimetric/Fluorometric Assay Kit (cat no. MAK329, Sigma-Aldrich, St. Louis, MO, USA), ATP Determination Colorimetric/Fluorometric Kit (ab83355, Abcam, Boston, MA, USA) and NADP/nicotinamide adenine dinucleotide phosphate (NADPH) Quantitation Kit (cat no. K347-100, Biovision, Osaka, Japan) were used for evaluating energy metabolism following the manufacturer's instructions.

Seahorse Assay

Extracellular acidification rates (ECAR) and cellular oxygen consumption rates (OCR) were monitored in real-time using the Seahorse XF96 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA, USA). To measure glycolysis and mitochondrial respiration, the media were replaced with XF media 1 hour before the experiment. Following a previously described protocol [22], a mixture of 10 mM glucose, 2.5 µM oligomycin, and 50 µM 2-deoxy-D-glucose (2-DG) was prepared in XF media according to the manufacturer's instructions for ECAR measurement. Furthermore, for OCR measurement, a mixture of 2.5 µM oligomycin, 1 mM FCCP, and 0.5 µM rotenone/antimycin A (rota-AA) was diluted in XF media as per the manufacturer's instructions.

Statistical Analysis

Quantitative data were presented as the means ± SEM (n = 3) unless otherwise specified. Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA, USA). Multiple group comparisons were performed using one-way ANOVA, followed by Tukey's post hoc test, and a comparison between the two groups was analyzed utilizing Tukey's test. Significant differences were determined at $p < 0.05$.

Results

NPM1 was Overexpressed in LUAD Cell Lines

RT-qPCR analysis showed that the mRNA levels of *NPM1* were significantly higher in LUAD cell lines compared to the normal lung cell lines BEAS-2B (Fig. 1A, $p < 0.001$). Similarly, the protein levels of *NPM1* were elevated in LUAD cell lines compared to the normal lung cell lines BEAS-2B (Fig. 1B,C, $p < 0.05$).

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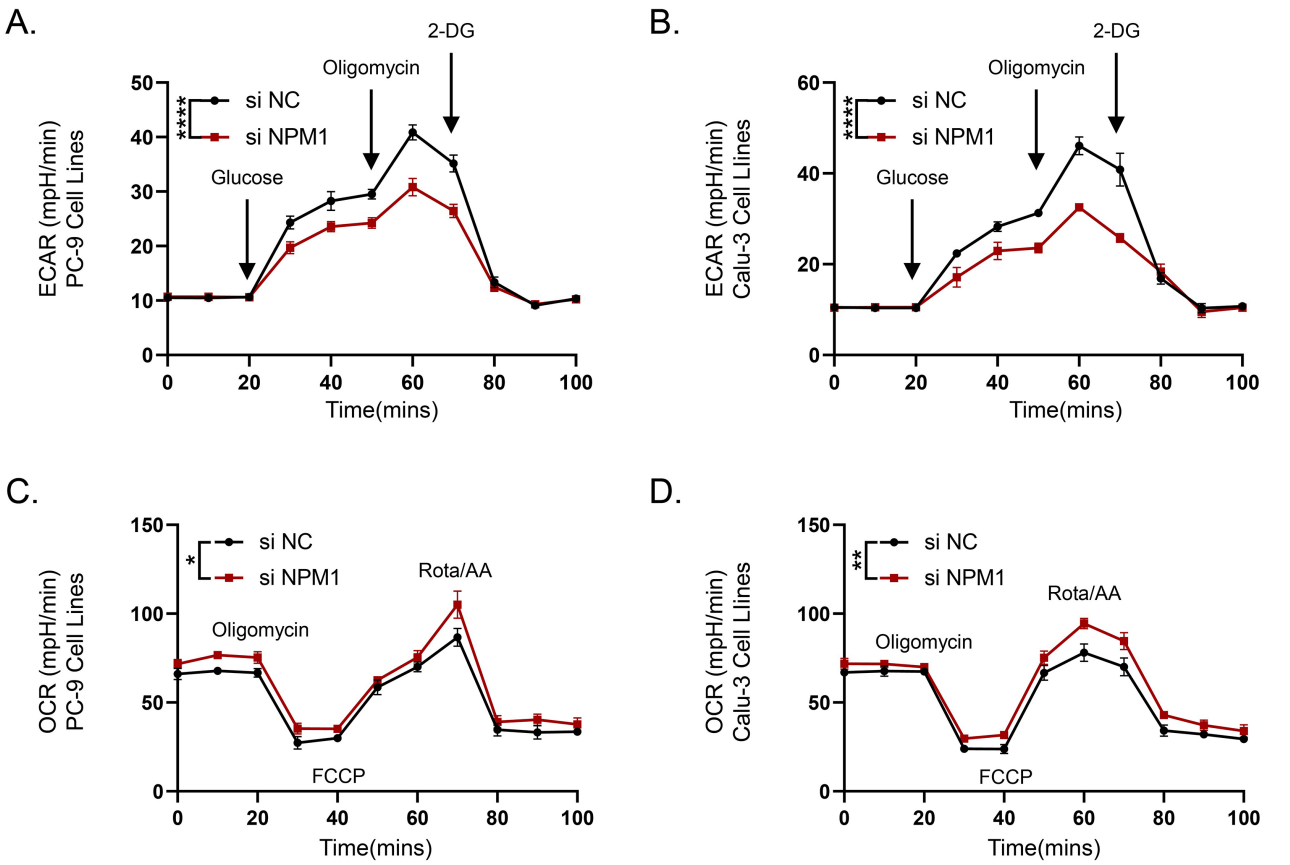

Fig. 5. The impact of *NPM1* silencing on oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) levels. (A,B) ECAR level detection. (C,D) OCR level detection. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, compared to the si-NC treated cell lines. n = 3.

Evaluation of Glycolytic Energy Metabolism in LUAD Cell Lines

The assessment of energy metabolism in LUAD cell lines revealed higher glucose uptake, elevated extracellular lactate levels, and reduced ATP production levels compared to the normal lung cell line BEAS-2B. These observations suggested that the energy metabolism pathway in these LUAD cell lines was primarily glycolytic (Fig. 2A–C, $p < 0.05$). Additionally, to investigate the role of *NPM1* in glycolysis, we constructed three vectors for silencing *NPM1* gene expression, as shown in Fig. 2D,E ($p > 0.05$). Among them, si-*NPM1*#3 showed the strongest gene-silencing effect. Hence, we used this vector for subsequent analysis.

NPM1 Silencing Suppressed the Proliferation, Motility, and Promoted Apoptosis in LUAD Cell Lines

To evaluate the biological functions of *NPM1* in LUAD cells, we transfected cells with selected si-*NPM1* and si-NC and performed cell growth, migration, invasion, and apoptosis analyses. The CCK-8 assay revealed a significant reduction in OD value for the si-*NPM1* group compared to the NC-mimic group at 72 hours ($p < 0.01$), indicating that si-*NPM1* potentially inhibited LUAD cell proliferation (Fig. 3A,B). Furthermore, Transwell migration and invasion assays revealed a lower migration rate in PC-9 and Calu-3 cells treated with si-*NPM1* compared to the si-NC mimic group ($p < 0.05$), suggesting that *NPM1* promoted the horizontal mobility of LUAD cells (Fig. 3C–F, $p < 0.01$). Additionally, we also observed the apoptosis ratio in si-*NPM1*-treated PC-9 and Calu-3 cell lines. After silencing *NPM1*, the apoptosis ratios were significantly increased in these two cell lines relative to the si-NC group cell line (Fig. 3G–J, $p < 0.01$). Collectively, these findings revealed the crucial role of *NPM1* in the biological activities of the LUAD cell line.

NPM1 Silencing Reduced Glycolysis in LUAD Cell Lines

After si-*NPM1* treatment, we observed declined glucose uptake and lactate production, and increased ATP generation and NADP+/NADPH ratio in PC-9 and Calu-3 cell lines (Fig. 4A–H, $p < 0.05$). Si-*NPM1* also reduced ECAR, demonstrating overall glycolytic flux and enhanced OCR (an indicator of mitochondrial respiration) (Fig. 5A–D, $p < 0.05$). These findings suggest that *NPM1* promotes glycolysis even in the presence of oxygen in LUAD cells.

Discussion

Since Otto Warburg discovered that cancer cells metabolize higher amounts of glucose through glycolysis, even in oxygen-rich environment, a phenomenon known as the Warburg effect [23], study has investigated aberrant glycolytic metabolism across different cancers to identify novel targets for tumor therapies [24]. LUAD is the main subtype of lung cancer. Previous studies have observed abnormal glycolysis in LUAD and identified specific LncRNAs or genes that promote LUAD development by enhancing glycolysis [10,12]. In this study, we explored the role of *NPM1* in LUAD progression based on the observations from a previously published bioinformatics article [18]. Our experimental results demonstrate that *NPM1* shows elevated expression in LUAD cell lines, enhancing LUAD progression through the glycolytic pathway.

NPM1 is a nucleocytoplasmic shuttle protein that fulfills numerous cellular functions, including ribosomal biogenesis, chromatin reshaping, genome stability, cell cycle transformation, and apoptosis [25]. Overexpression or mutation, have been implicated in cancer development and tumor progression, associated with poor prognosis [26–28]. In this study, we explored the impact of *NPM1* on LUAD progression. Similar to previous bioinformatics data [18], we found *NPM1* overexpression in LUAD cell lines. Studies have demonstrated the potential role of *NPM1* in proliferation [29], migration, invasion [30], or metastases [31] in lung cancer. To supplement and enhance our understanding, we also validated the role of *NPM1* in the proliferation, migration, and invasion of LUAD cell lines. Furthermore, we revealed its function in the apoptosis of LUAD cell lines.

NPM1 facilitates the progression of LUAD through the EGFR/MAPK signaling pathway [30]. Moreover, gene set enrichment analysis indicated an association between *NPM1* and aerobic glycolysis in LUAD [18]. There is abundant evidence supporting the role of glycolysis in cancer progression, rendering it a promising therapeutic target for cancer treatment [32]. For instance, Yang J *et al*. [33] elucidated enhanced glycolysis in pancreatic cancer metastasis. Similarly, Zhao *et al*. [34] reported that LncRNA MIR17HG promotes colorectal cancer liver metastasis by regulating positive feedback relevant to glycolysis.

Additionally, significant changes in major enzymes of the aerobic glycolytic pathway have been observed in breast cancer [35]. Studies exploring glycolysis in lung cancer have also been conducted. For example, the notch1/TAZ axis has been observed to strengthen aerobic glycolysis and immune escape in lung cancer [36]. Additionally, recombinant oncolytic adenovirus containing apoptin- Ad-apoptin could kill cancer cells, with its ability to inhibit glycolysis in lung cancer cells by mediating the AMPK/mTOR signaling pathway [37]. Based on the bioinformatics prediction results and the significance of glycolysis in various cancers, this study investigated the role of *NPM1* in aerobic glycolysis in LUAD. Our findings demonstrated an increase of glycolysis in LUAD cell lines, which was reduced following *NPM1* silencing. This observation indicated that *NPM1* plays a crucial role in developing LUAD by regulating glycolysis. However, the mechanism underlying the involvement of NPM1 in LUAD development requires further investigation, which is the direction of our future research.

Conclusion

In summary, *NPM1* contributes to the proliferation, migration, invasion, and apoptosis of LUAD through the glycolytic pathway, indicating its potential as a new drug target and therapeutic strategy to benefit a broader range of LUAD patients.

Abbreviations

ECAR, extracellular acidification rates; LUAD, lung adenocarcinoma; *NPM1*, Recombinant Nucleophosmin 1; OCR, oxygen consumption rates; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; rota-AA, rotenone/antimycin A; WB, western blotting; 2-DG, 2-deoxy-D-glucose.

Availability of Data and Materials

All data included in this study are available upon request by contact with the corresponding author. Available at NTC (zn002749@whu.edu.cn).

Author Contributions

All authors contributed to this present work: NTC and PFJ designed the study. XFZ and MX collected and analyzed data. NTC and PFJ drafted the manuscript. XFZ and PFJ reviewed and revised the manuscript. All authors have been involved in revising it critically for important intellectual content. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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