

Original Article

Mitochondrial calcium uniporter as a biomarker in lung adenocarcinoma: regulation of the immune microenvironment, apoptosis, cell cycle and mitophagy

Yihao Liu^{1,4,5*}, Zexia Zhao^{1,2*}, Hua Huang^{1,2*}, Chenxuan Zhao^{3*}, Zhixuan Duan^{1,2}, Min Wang^{1,2}, Chen Ding^{1,2}, Wenhao Zhao^{1,2}, Yanzhan Liu^{1,2}, Hongbing Zhang^{1,2}, Yongwen Li^{1,2}, Wenjun Meng⁴, Yin Li^{5#}, Xiaobin Shang^{5#}, Jun Chen^{1,2#}, Haoling Zhang^{6#}, Honglin Zhao^{1,2#}

¹Department of Lung Cancer Surgery, Tianjin Medical University General Hospital, Tianjin 300052, The People's Republic of China; ²Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Tianjin Lung Cancer Institute, Tianjin Medical University General Hospital, Tianjin 300052, The People's Republic of China; ³Tianjin Yizhong Middle School, Tianjin 300051, The People's Republic of China; ⁴Department of Pain Management, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, The People's Republic of China; ⁵Department of Thoracic Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100000, The People's Republic of China; ⁶Department of Biomedical Sciences, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Kepala Batas 13200, Penang, Malaysia. *Equal contributors. #Equal contributors and co-corresponding authors.

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Abstract: The mitochondrial calcium uniporter (MCU) complex is essential for maintaining mitochondrial calcium homeostasis and regulating cellular metabolism, apoptosis, proliferation, and mitochondrial quality control. Although MCU has been implicated in multiple malignancies, its biological role and regulatory mechanisms in lung adenocarcinoma (LUAD), a major subtype of non-small cell lung cancer (NSCLC), remain insufficiently defined. In this study, MCU expression was analyzed using pan-cancer and LUAD datasets from TCGA, GEO, UALCAN, and tissue microarray cohorts. GO, KEGG, and GSEA were performed to explore MCU-associated biological pathways, while ssGSEA, CIBERSORT, and TIDE algorithms were used to evaluate immune infiltration and predicted immunotherapy response. In vitro assays, including CCK-8, EdU, colony formation, Transwell assays, flow cytometry, qRT-PCR, Western blotting, immunofluorescence staining, JC-1 staining, and reactive oxygen species (ROS) detection, were conducted to assess the effects of MCU on LUAD cell behavior, mitophagy, and mitochondrial function. MCU was significantly upregulated in LUAD tissues and cell lines compared with normal controls. High MCU expression was associated with reduced immune cell infiltration, decreased immune checkpoint and HLA gene expression, and lower predicted sensitivity to immunotherapy. Functionally, MCU knockdown markedly inhibited LUAD cell proliferation, migration, and invasion, promoted apoptosis, and induced G1-phase cell cycle arrest, accompanied by increased p21 and cleaved caspase-3 expression and decreased CDK4 and Cyclin D1 expression. Conversely, MCU overexpression enhanced malignant phenotypes and suppressed apoptosis. Mechanistically, GSEA indicated that MCU was closely associated with mitophagy-related pathways. Further validation showed that MCU knockdown reduced PINK1 and PRKN expression, decreased mitochondrial LC3B accumulation, and weakened LC3B-mitochondria co-localization, indicating impaired mitophagy. MCU depletion also caused mitochondrial membrane potential dissipation and increased intracellular ROS accumulation. Collectively, these findings suggest that MCU promotes LUAD progression by remodeling the tumor immune microenvironment, enhancing malignant cellular behaviors, inhibiting apoptosis, and maintaining mitophagy-dependent mitochondrial homeostasis, highlighting MCU as a potential prognostic biomarker and therapeutic target in LUAD.

Keywords: Mitochondrial calcium uniporter (MCU), lung adenocarcinoma (LUAD), immune microenvironment, apoptosis, cell cycle, mitophagy

Introduction

Lung cancer is one of the most frequently occurring malignant diseases in the world at present, and the two principal types are small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) [1]. NSCLC is responsible for about 85% of all cases, and it is mainly histologically lung adenocarcinoma (LUAD) [2]. The different ways to treat LUAD include surgery, chemotherapy, immunotherapy, targeted therapy and combinations of the above [3-5]. However, the death rate of people with advanced LUAD is also less than 15% [6]. Therefore, in-depth studies of the causes of LUAD and the discovery of new biomarkers are urgently needed [7, 8].

The mitochondrial calcium uniporter (MCU) is a multi-protein complex composed of several individual units, such as MCU, the MICU family, MCUb, EMRE, MCUR1, and SLC25A23 [9]. Regulation of mitochondrial calcium homeostasis by this complex is required for the transport of cytosolic calcium into the mitochondrial matrix and thus maintains normal cell function. Therefore, MCU is needed for the energy metabolism in cells, oxidative stress response, and regulation of the cell cycle [10-13]. Although some research has been conducted recently, the exact reasons for this effect are not yet known [9]. MCU promotes the proliferation and metastasis of breast cancer cells by increasing mitochondrial calcium influx (mCa^{2+}) [14]. On the other hand, in other circumstances, such as treatment with RY10-4 derivatives, MCU expression is enhanced; as a result, there is mCa^{2+} overload, mitochondrial dysfunction, and subsequently, apoptosis of tumor cells [15]. MCU overexpression in colorectal cancer enhances the stabilisation of mitochondrial transcription factor A (TFAM) through mCa^{2+} regulation and promotes tumor growth [16]. Pancreatic ductal adenocarcinoma enhances the metastatic potential of MCU by increasing the production of reactive oxygen species (ROS), damaging Keap1 through oxidation, and thus activating the Nrf2 antioxidant pathway [17]. MCU also promotes the growth and spread of oral squamous cell carcinoma by activating Nrf2 in the same way [18]. MCU promotes the growth of gastric cancer and angiogenesis through the regulation of VEGF and epithelial-mesenchymal transition (EMT) [19]. MCU is also related to several other diseases of non-

cancerous origin, such as damage to the myocardium, heart failure, and type 2 diabetes, which are caused by changes in intracellular calcium (iCa^{2+}) and mitochondrial dynamics [20-22]. Based on the above research results, MCU is now considered potential therapeutic targets for both cancer and non-cancerous diseases related to the regulation of mCa^{2+} . MCU is also promising candidates for diagnostic biomarkers in precision medicine.

Dysregulation of the cell cycle and evasion of apoptosis are features of cancer that drive the start and spread of tumors, and reduce drug efficacy [23-27]. Regulation of the cell cycle for regular division of cells; elimination of damaged and abnormal cells through apoptosis to maintain tissue homeostasis. Perturbations in the above processes have been shown to be closely related to the development of some cancers, particularly NSCLC. Aberrant expression or dysfunction of cell cycle regulators, such as cyclin-dependent kinases (CDKs), cyclins and their inhibitors (e.g., p21), is frequently found in NSCLC [28, 29]. At the same time, damage to the apoptotic pathway can lead to an increased number of cancer cells, which are able to withstand stress and avoid recognition by the immune system [30-32].

Mitophagy is a specific type of autophagy that selectively degrades damaged or non-functional mitochondria and maintains the quality of mitochondria [33]. Under the conditions of oxidative stress, hypoxia, nutrient deficiency and loss of mitochondrial membrane potential, damaged mitochondria are identified and taken up by autophagosomes, which then fuse with lysosomes for breakdown [34]. The above process will maintain cell homeostasis and adapt metabolically. Based on the above studies, mitophagy is associated with the development of lung cancer. MCU is a typical regulator of mitochondrial Ca^{2+} uptake and mitochondrial homeostasis that has also recently been linked to the control of mitophagy. MCU-mediated calcium signalling can modify mitochondrial membrane potential, ROS generation and other features of the mitochondrial metabolic state to influence mitophagy [35]. Thus, mitophagy may be a possible mechanism by which abnormal MCU expression drives the progression of LUAD. However, the exact molecular mechanisms of collaboration between MCU and mitophagy in LUAD have yet to be fully identified.

Differential expression analysis of MCU in normal and tumor tissue was performed on public databases in this study. Function-enrichment analysis was also performed to examine the biological effects and regulatory mechanisms of MCU in LUAD, as well as alterations in immune cell infiltration, immunotherapy responses, apoptosis and cell cycle regulation. Based on the above results, we have identified potential oncogenic functions of MCU in LUAD and propose new diagnostic indicators for this illness.

Materials and methods

Data acquisition and processing

LUAD-related data have been obtained from The Cancer Genome Atlas (TCGA) at <https://portal.gdc.cancer.gov/>. After removing duplicate entries and incomplete clinical data, gene expression profiles and clinical data from 489 LUAD patients and 59 adjacent normal tissue samples were retained. To obtain the GSE32863 and GSE116959 datasets as external validation sets for differential expression analysis, the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) database was used. GSE32863 contains 58 tumor and 58 normal tissue samples, and GSE116959 has 57 tumor and 11 normal samples [36]. Differential expression of MCU in numerous cancers has been investigated using data from the UALCAN database (The University of Alabama at Birmingham Cancer Data Analysis Portal) [37]. Immunohistochemistry images of MCU in normal and tumor tissues were obtained from the Human Protein Atlas (HPA; <https://www.proteinatlas.org/>) [38]. RNA sequencing data of 33 pairs of LUAD tumor and adjacent normal tissues were also obtained from the Department of Thoracic Surgery, Tianjin Medical University General Hospital (TMU) [39]. All data processing and plotting were done with R (version 4.4.4).

Single cell transcriptome analysis

The scRNA-seq dataset GSE127465 was downloaded from the GEO database [40]. Raw scRNA-seq data were processed and analysed in the Seurat R package. Low-quality cells were excluded by not including those with fewer than 500 or more than 5,000 detected genes, as well as cells with over 10% mitochondrial gene expression. Harmony R package was used to correct for batch effects. Annotation of cell

types was carried out based on the above standard marker genes.

GO/KEGG/GSEA functional enrichment analysis of MCU-related genes

Pearson correlation analysis in R was conducted on TCGA data to find MCU-associated genes with a correlation coefficient ≥ 0.3 . Functional enrichment analysis of the clusters was performed using the clusterProfiler package in R to explore potential signalling pathways and pathogenic mechanisms, such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. Gene set enrichment analysis (GSEA) was also used to determine whether the changes in gene expression for MCU were related to particular biological pathways at the level of gene sets [41].

Immune infiltration and immunotherapy response

Four hundred and eighty-nine LUAD samples were split into a high-expression group and a low-expression group according to the MCU expression level. The immune activity of the 28 immune cell types and the overall immune score were assessed using single-sample Gene Set Enrichment Analysis (ssGSEA) [42]. Differential immune expression was analysed with the 'ggpubr' R package. In addition, differences in expression for immune checkpoint genes, Human Leukocyte Antigen (HLA) genes, and leukocyte-mediated cytotoxicity-related genes between the high-expression group and the low-expression group were also investigated. The proportions of different types of immune cells in the two groups were analysed by CIBERSORT [43]. Using the TIDE website (<http://tide.dfci.harvard.edu/>), gene expression data were entered to calculate tumor Immune Dysfunction and Exclusion (TIDE) scores for the investigation of immune evasion and immunotherapy response.

Cell culture and transfection

The human bronchial epithelial cell line BEAS-2B and five frequently used LUAD cell lines (HCC827, H1975, A549, PC9 and H1299) were all obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI-1640 or DMEM (Gibco, NY, USA) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-strepto-

mycin. At 37°C, 5% CO₂-humidified air was used to keep the culture, and fresh medium was added every two or three days. Seed cells in 6-well plates at a density of 5×10^5 cells/well, and transfect them when cell confluence reaches 60%-70% after 24 hours. Lipofectamine™ 2000 (Thermo Fisher, USA) was used to perform transfection of si-MCU and plasmids according to the manufacturer's instructions [44].

Quantitative real-time PCR (qRT-PCR)

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used for total RNA extraction, and then PrimeScript RT reagent kit (TaKaRa, Beijing, China) was employed to synthesize cDNA. Quantitative real-time PCR was used to measure the mRNA abundance of MCU and β -actin; β -actin served as the reference gene for internal normalization, and a SYBR Green-based master mix was employed for qRT-PCR. Each 20 μ L reaction mixture contained 10 μ L of 2 $\times$ SYBR Green master mix, 2 μ L of forward primer (10 μ M), 2 μ L of reverse primer (10 μ M), 2 μ L of cDNA template, and nuclease-free water to a final volume of 20 μ L. Amplification conditions are as follows: Initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. All qRT-PCR experiments were independently performed in triplicate. Calculate relative mRNA expression levels by the $2^{-\Delta\Delta Ct}$ method [45]. The primer sequences of the qRT-PCR primers are given in [Supplementary Table 1](#).

Western blotting

Add RIPA lysis buffer with Phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitors (Beyotime Biotechnology, China) to extract the total proteins, and then measure them by BCA protein assay. Equal amounts (30 μ g) of protein were separated by SDS-PAGE and transferred onto PVDF membranes. Block membranes with 5% BSA for 1 hour, then overnight at 4°C add primary antibodies ([Supplementary Table 2](#)). Wash, then add a suitable HRP-conjugated mouse or rabbit secondary antibody for 1 hour, and finally use enhanced chemiluminescence to observe the results.

Cell viability assay

At a concentration of 1×10^4 cells/well, cells were added to 96-well plates. At 0, 24, 48 and

72 hours, CCK-8 reagent (Beyotime Biotechnology, China) was added and the absorbance was determined by a microplate reader. All experiments were independently repeated three times.

EdU cell proliferation assay

At 8×10^3 cells/well, cells were added to a 96-well plate. After 24 hours, 50 μ M EdU (RiboBio, China) was added and incubated at 37°C for 2 hours. Fix, stain and then perform EdU detection on the cells using a kit by RiboBio (China) for fluorescence microscopy. The number of EdU-positive (red-stained) cells was divided by the number of Hoechst 33342-stained (blue) nuclei to obtain the proliferation rate.

Colony formation assay

Cells (500/well) were added to a 6-well plate and incubated for 14 days. Three washes in PBS were performed on the colonies, followed by 40-minute fixation with 4% paraformaldehyde and staining with crystal violet solution (Beyotime Biotechnology, China) for 40 minutes. Wash with PBS and then count the number of colonies using ImageJ software.

Transwell assay

Transwell plates (BD Biosciences, New York, USA) were used for the migration assay without Matrigel. A total of 5×10^4 cells were seeded in the upper wells, and 600 μ L of complete medium containing 20% FBS was added to the lower wells. After 24 hours, the migrated cells were fixed with paraformaldehyde and stained using 600 μ L of crystal violet solution.

Thaw Matrigel at 4°C and dilute it 1:8 in serum-free medium for the invasion assay. Fifty microliters of the diluted Matrigel were added to the upper chambers, incubated at 37°C for 2 hours to allow for gelation, and then the cells were seeded. After 48 hours, the invaded cells were fixed and stained, then observed through a microscope after drying.

Apoptosis and cell cycle analysis

Harvest tumor cells by trypsinization and wash twice in PBS. Centrifuge at 4°C, add 300 μ L of 1 $\times$ binding buffer, and then incubate with Annexin V-FITC at room temperature for 20 minutes. PI (BD Biosciences, USA) was then added

and incubated for 10 minutes prior to flow cytometry analysis of apoptosis. For cell cycle analysis, overnight at -20°C, the cells were fixed in 80% ethanol and then stained with 500 µL of PI at room temperature for 30 minutes in the dark before being analysed by flow cytometry.

Immunofluorescence staining

Cells were added to a sterile dish and grown at a high density. After 15 minutes of fixation in 4% paraformaldehyde, 0.5% Triton X-100 was used to permeabilise the cells for 10 minutes, followed by a block of 1% BSA for 40 minutes to reduce non-specific binding. Then, the samples were added to the indicated primary antibodies for one night at 4°C. After thorough washing, the cells were added to fluorophore-conjugated secondary antibodies for 1 h at room temperature in the dark. Counterstain with 4',6'-diamidino-2-phenylindole (DAPI), and then mount the coverslip in an antifade mounting medium. Fluorescence images were acquired using an Olympus confocal microscope (Tokyo, Japan).

Assessment of mitochondrial membrane potential

JC-1 assay kit (MedChemExpress, New Jersey, USA) is to be used for the mitochondrial membrane potential experiment. Briefly, the treated cells were added to 2 µM JC-1 dye in serum-free medium for 20 minutes at 37°C in a humidified incubator with 5% CO₂. Incubate for a short time, then wash the cells twice with PBS to eliminate the residual dye, and immediately conduct flow cytometry experiments on an Agilent Technologies flow cytometer (Santa Clara, CA, USA). JC-1 fluorescence was observed at an excitation wavelength of 488 nm, and both green fluorescence (corresponding to JC-1 monomers) and red fluorescence (corresponding to JC-1 aggregates) were collected at 530 nm and 590 nm, respectively.

Detection of reactive oxygen species

ROS inside the cell were measured with a ROS assay kit (Beyotime). After the above treatments, cells were added to 10 µM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in serum-free medium for 20 minutes at 37°C in the dark. Add PBS + 0.1% azide to wash away the probe after the incubation. Fluorescence

intensity was then determined by flow cytometry on a flow cytometer (Agilent Technologies, Santa Clara, CA, USA), and intracellular ROS levels were quantitatively assessed.

Statistical analysis

GraphPad Prism, R (version 4.4.4) and ImageJ were used for statistical analysis. The mean ± standard deviation (SD) of the data are shown below. For the comparison of two groups, a Student's t-test or a Wilcoxon rank-sum test was selected. A one-way analysis of variance followed by an appropriate post-hoc test was used to compare several groups. Repeated-measures ANOVA was used for the experiments that measured data at different times. All the experiments were repeated three or more times. Statistical significance was set at $P < 0.05$, and the corresponding levels of significance were as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Databases and online tools used

TCGA: <https://portal.gdc.cancer.gov/>; GEO: <https://www.ncbi.nlm.nih.gov/geo/>; TIDE: <http://tide.dfci.harvard.edu/>; UALCAN: <https://ualcan.path.uab.edu/>; HPA: <https://www.protein-atlas.org/>.

Results

Differential expression of MCU across pan-cancers and upregulation in LUAD

The general study design and analysis process of MCU are shown in [Supplementary Figure 1A](#). The tissue-wide expression pattern of MCU in human organs is shown in [Supplementary Figure 1B](#), and it can be seen that MCU is widely distributed in all parts of the body. Using the HPA database, the subcellular locations of MCU were also identified ([Supplementary Figure 1C](#)). Protein expression analysis in the UALCAN database shows that most tumor types have increased MCU levels (**Figure 1A**), and lung cancer is among those with a relatively large increase ($P < 0.001$; **Figure 1B**). Consistently, mRNA expression analysis in TCGA data also shows that MCU is overexpressed in many cancers compared with the corresponding normal tissues (**Figure 1C**), and this includes a significant increase in NSCLC ($P < 0.001$; **Figure 1D**). Pairwise sample analysis was also conducted

MCU as a biomarker in lung adenocarcinoma

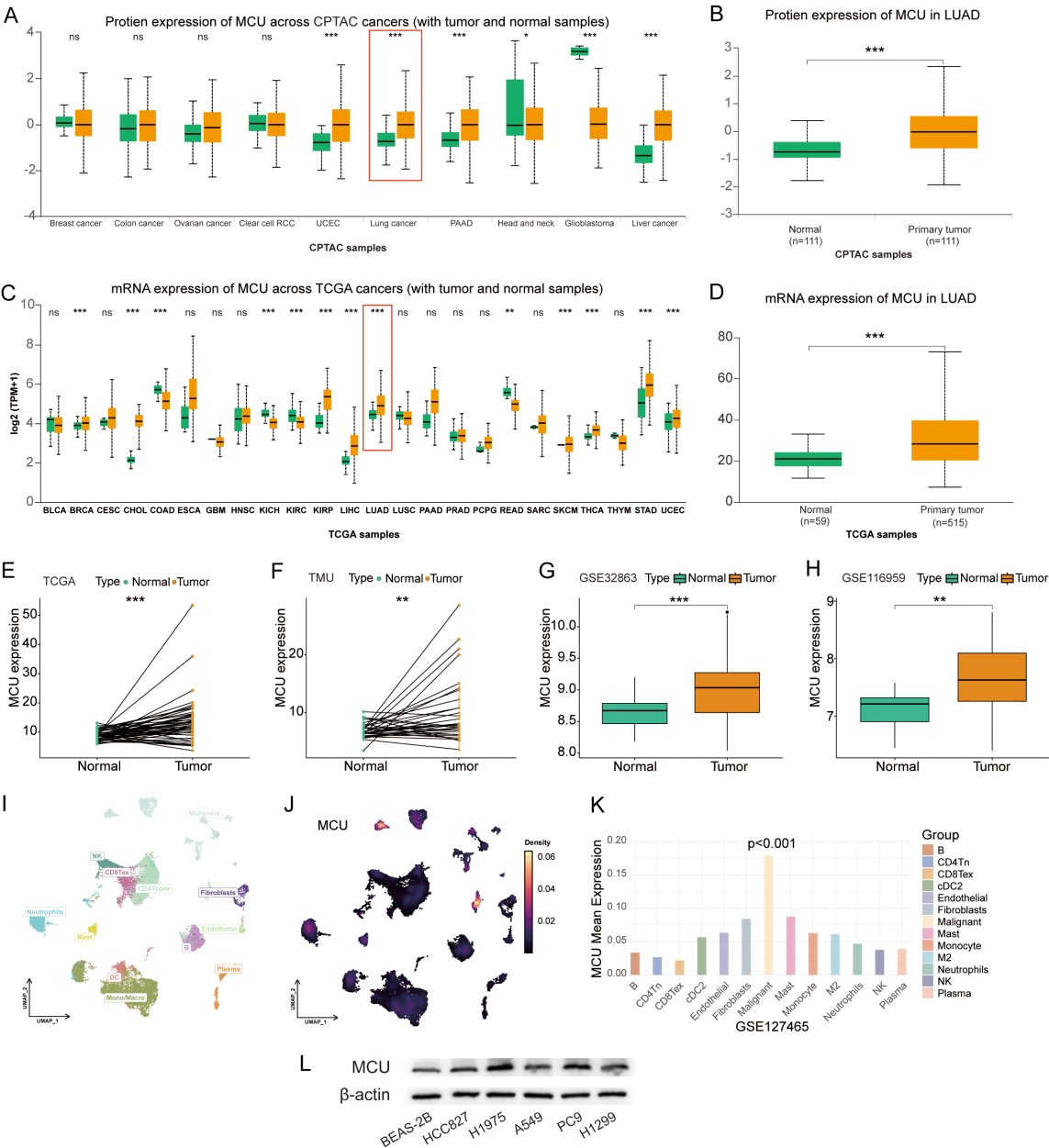


Figure 1. Expression of MCU across pan-cancers and in LUAD. A. Protein expression of MCU across various cancers based on the UALCAN database; B. MCU protein expression in lung cancer versus adjacent normal tissues using the UALCAN database; C. mRNA expression of MCU across pan-cancers from the TCGA dataset; D. Comparison of MCU mRNA expression between LUAD and normal tissues; E, F. Paired analysis of MCU expression in tumor versus adjacent normal tissues using TCGA and TMU cohorts; G, H. Validation of MCU differential expression in tumor and normal tissues based on GSE32863 and GSE116959 datasets; I. UMAP visualization of cell subpopulation clustering; J. Single cell level density distribution of MCU expression across different cell subpopulations; K. Expression levels of MCU in each identified cell subpopulation; L. MCU expression levels in various lung cancer cell lines.

on TCGA and TMU data, showing that the expression of MCU was significantly higher in tumor tissue compared with that in corresponding normal tissue (Figure 1E, 1F). The above results have been validated in two GEO datas-

ets, GSE32863 and GSE116959, and are in agreement (Figure 1G, 1H). MCU was relatively abundant in malignant tumor cells in single-cell transcriptomics analysis and showed a significant increase (P < 0.001; Figure 1I-K).

Immunohistochemistry images of MCU expression in LUAD tissues were also obtained from the HPA database, and it was found that the protein level in the tumor tissue exceeded that in normal tissue ([Supplementary Figure 1D](#)). Western blot analysis also shows that in all five LUAD cell lines (HCC827, H1975, A549, PC9 and H1299), the expression of MCU is relatively increased compared to the normal bronchial epithelial cell line BEAS-2B. Among them, H1975 and PC9 had the highest expressions, and A549 and H1299 were relatively low ([Figure 1L](#) and [Supplementary Figure 2A](#)).

Functional enrichment analysis of MCU-related genes

Genes that are correlated with MCU by more than 0.3 were included in the functional enrichment analysis. GO enrichment analysis shows that the above genes are significantly associated with the following pathways: cell killing, regulation of immune effector process, leukocyte-mediated cytotoxicity, T cell-mediated immunity, execution phase of apoptosis and regulation of cell cycle phase transition ([Figure 2A](#)). KEGG pathway enrichment analysis shows that hematopoietic cell lineage and systemic lupus erythematosus were significantly enriched pathways ([Figure 2B](#)). GSEA further verified the above associations and presented biological processes, including cell death, regulation of the epithelial cell apoptotic process, immune effector function, and the mitotic cell cycle ([Figure 2C-G](#)). Based on the above results, it can be concluded that most of the MCU-related genes are involved in cytotoxicity, immune regulation, apoptosis and cell cycle control, and thus may promote the progression of LUAD.

Association between immune infiltration and MCU expression

To investigate the immune microenvironment associated with MCU expression, we have examined the abundance of immune cells in the high-MCU and low-MCU groups. ssGSEA analyses showed that most types of immune cells were significantly less numerous in the high-MCU group compared with the low-MCU group ([Figure 3A](#)). Similarly, the expression levels of immune checkpoint genes, HLA family genes, and leukocyte-mediated cytotoxicity-related genes were also significantly lower in

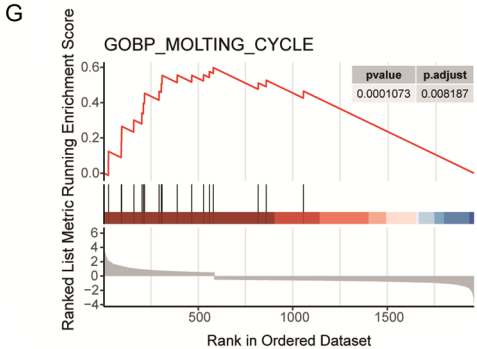
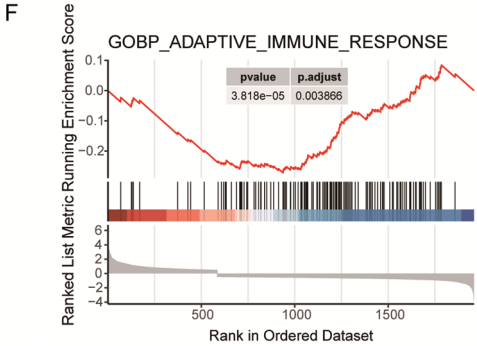
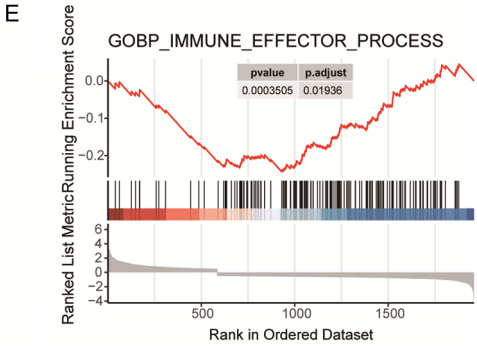
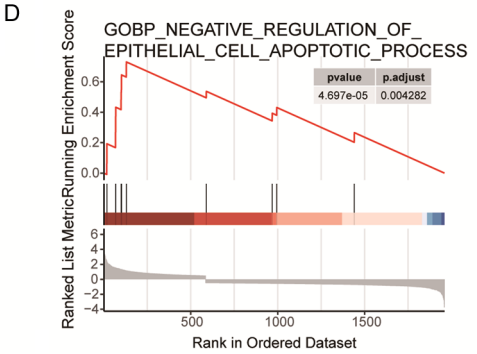
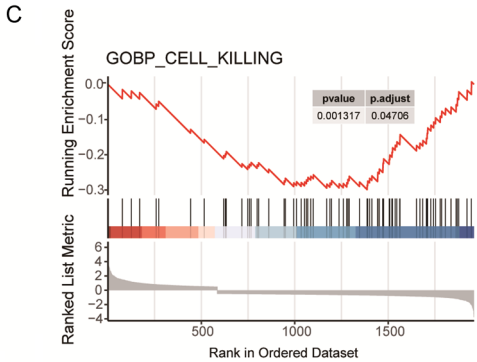
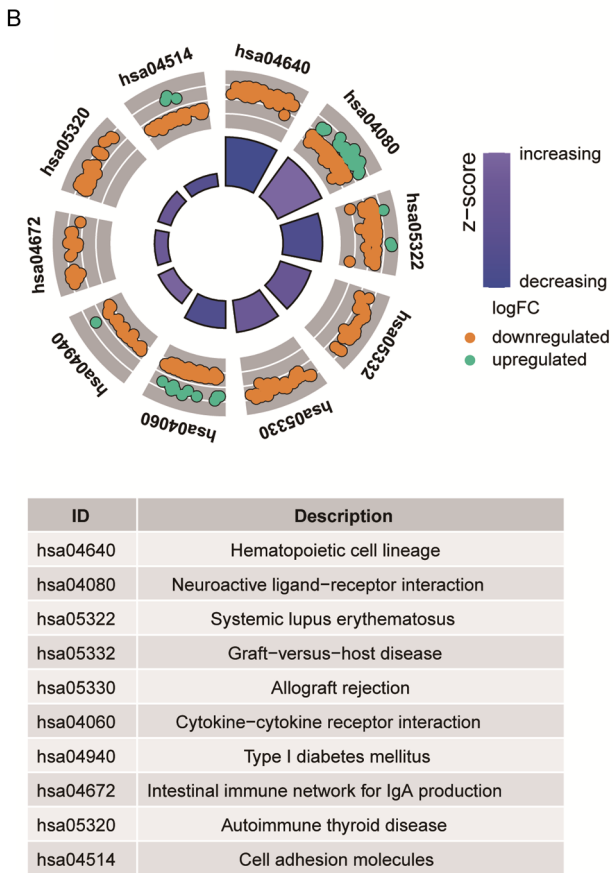
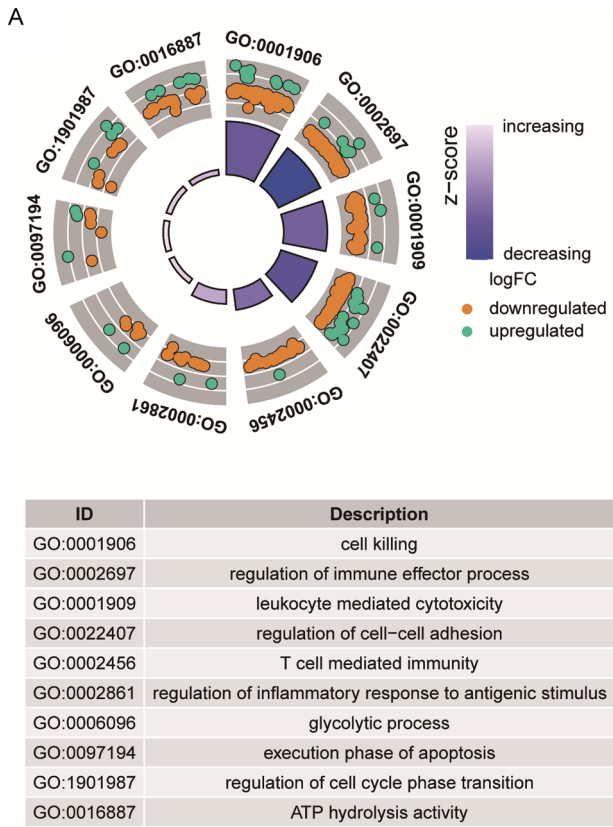
patients with high MCU expression ([Figure 3B-D](#)). Immune scores and TIDE (tumor Immune Dysfunction and Exclusion) scores were also computed. The high-MCU expression group had a significantly lower immune score ($P < 0.001$) and was therefore less immune. In addition, a high TIDE score in the high-MCU group was associated with more immune evasion and a higher risk of resistance to immunotherapy ([Figure 3E, 3F](#)). Rainbow bar chart of the proportion of different kinds of immune cells in CIBERSORT analysis ([Figure 3G](#)). The high-risk group (high-MCU) showed reduced response to immunotherapy and poor immune response ([Figure 3H](#)). Overall, high expression of the MCU is likely to reduce immune cytotoxicity, suppress antigen presentation and immune activation by inhibiting the recruitment of immune cells, and downregulate families of immunoregulatory genes (such as HLA), thereby promoting immune evasion and resistance in LUAD.

Effect of MCU on LUAD cell proliferation, migration, and invasion

To explore the effect of MCU on LUAD in a biological way, two cell lines with high MCU expression (H1975 and PC9) were used for siRNA-mediated knockdown ([Figure 4A, 4B](#) and [Supplementary Figure 2B](#)). CCK-8 assay data show that silencing MCU reduced cell viability significantly ([Figure 4C](#)). EdU incorporation assays also showed that MCU knockdown reduced the proliferation of both H1975 and PC9 cells ([Figure 4D, 4E](#)). To evaluate the effects on migration and invasion, a wound healing assay and Transwell assay were carried out. The results show that MCU significantly inhibited the migration and invasion of LUAD cells ([Figure 4F-I](#)).

On the other hand, MCU was overexpressed in A549 and H1299 cells, which have relatively low basal levels of MCU expression ([Figure 5A, 5B](#) and [Supplementary Figure 2C](#)). CCK-8, EdU, colony formation and Transwell assays all show that overexpressing MCU increases the proliferation, migration and invasion of these cell lines ([Figure 5C-I](#)). Together, the above results indicate that the MCU is necessary for the promotion of multiple malignant behaviours by LUAD cells, such as proliferation, migration and invasion.

MCU as a biomarker in lung adenocarcinoma



MCU as a biomarker in lung adenocarcinoma

Figure 2. Functional enrichment analysis of MCU-related genes. A. GO enrichment results of MCU-related genes; B. KEGG pathway enrichment; C-G. GSEA of MCU-associated biological processes.

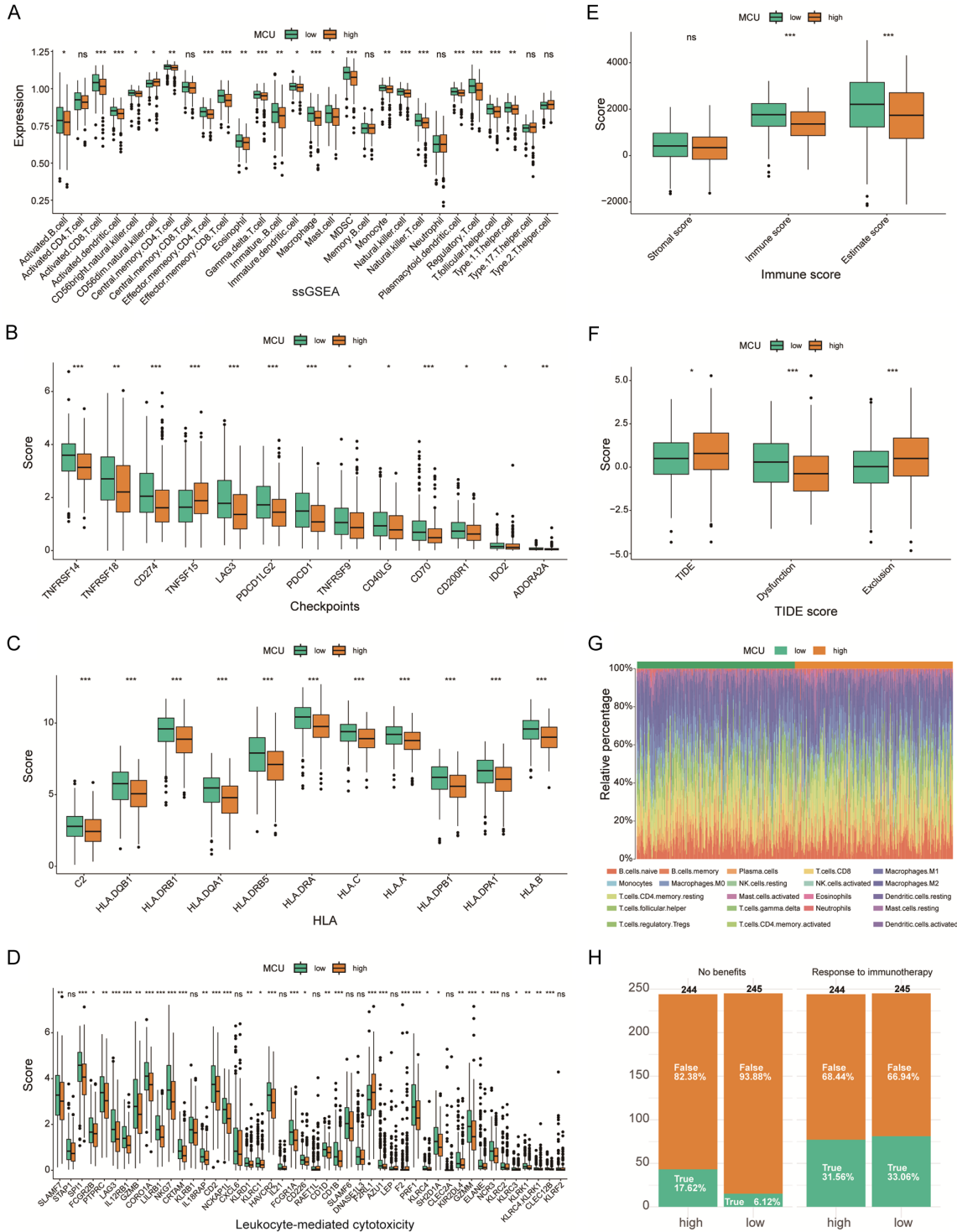


Figure 3. Tumor microenvironment and immunotherapy response in MCU high- and low-expression groups. A. Expression levels of 28 immune cell types between MCU high and low groups based on the ssGSEA algorithm; B. Expression of immune checkpoint gene family; C. HLA gene family expression; D. Expression of leukocyte-mediated cytotoxicity-related genes; E. Immune score comparison; F. TIDE scores; G. Immune cell infiltration proportion based on the CIBERSORT algorithm; H. Comparison of predicted immunotherapy benefit and responsiveness. *P < 0.05, **P < 0.01, ***P < 0.001; ns: not significant.

MCU as a biomarker in lung adenocarcinoma

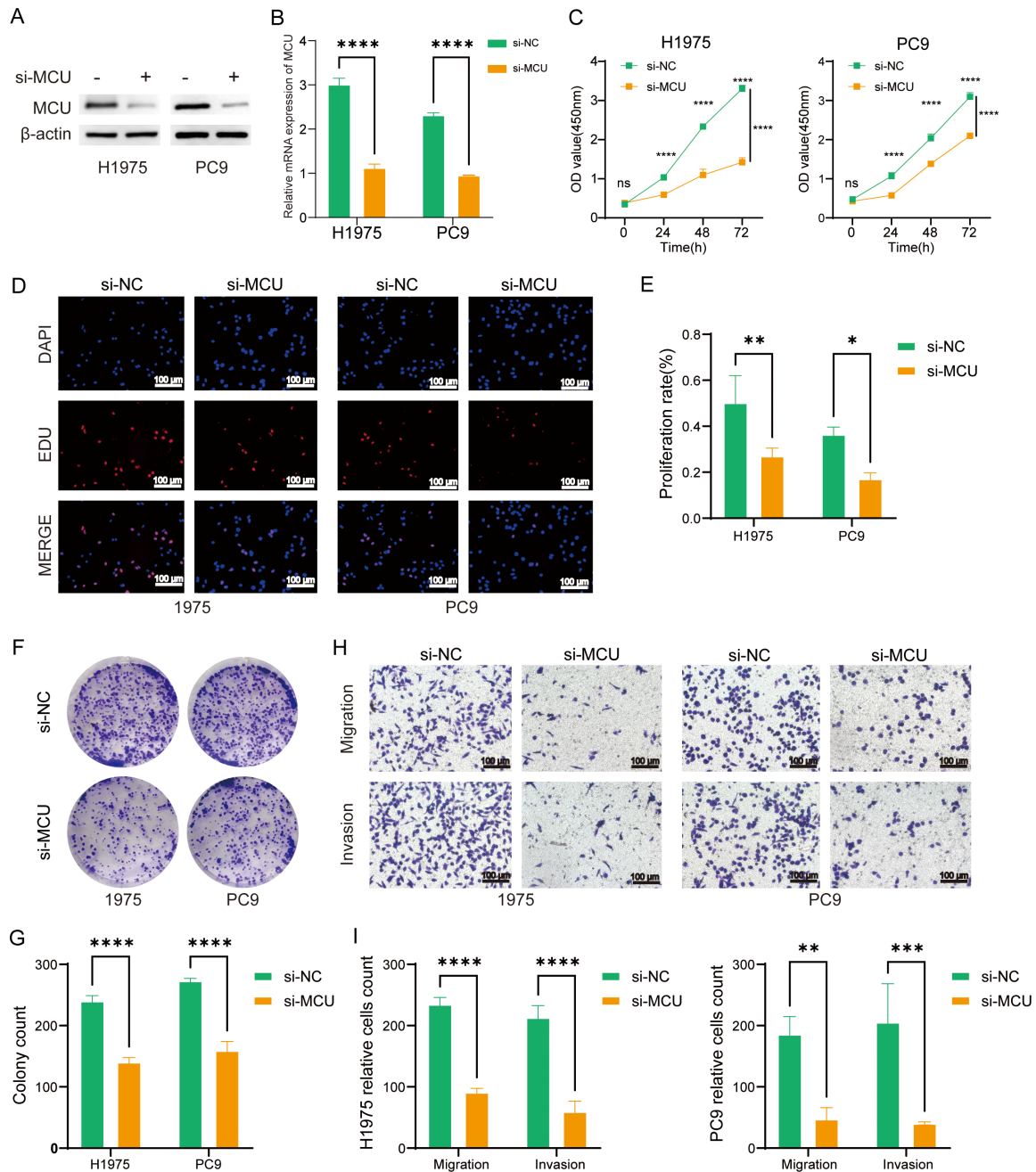


Figure 4. Knockdown of MCU suppresses proliferation, colony formation, migration, and invasion in LUAD cell lines H1975 and PC9. **A**, Western blot showing MCU protein levels after siRNA transfection in H1975 and PC9 cells, with β -actin as a loading control; **B**, qRT-PCR showing MCU mRNA expression after si-MCU transfection; **C**, Cell proliferation at 24, 48, and 72 hours measured by CCK-8 assay; **D**, **E**, EdU staining and quantification of cell proliferation; **F**, **G**, Colony formation assays in si-NC and si-MCU groups; **H**, **I**, Transwell assays evaluating migration and invasion capacity of H1975 and PC9 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns: not significant.

Effect of MCU on apoptosis and cell cycle in LUAD

In addition, we have studied how MCU regulate LUAD cell behaviour by exploring the pathways

of apoptosis and the cell cycle in previous enrichment analyses. Flow cytometry showed that knockdown of MCU increased the percentage of early apoptotic cells (Q3-2 + Q3-4) in H1975 and PC9 cells (**Figure 6A**), while overex-

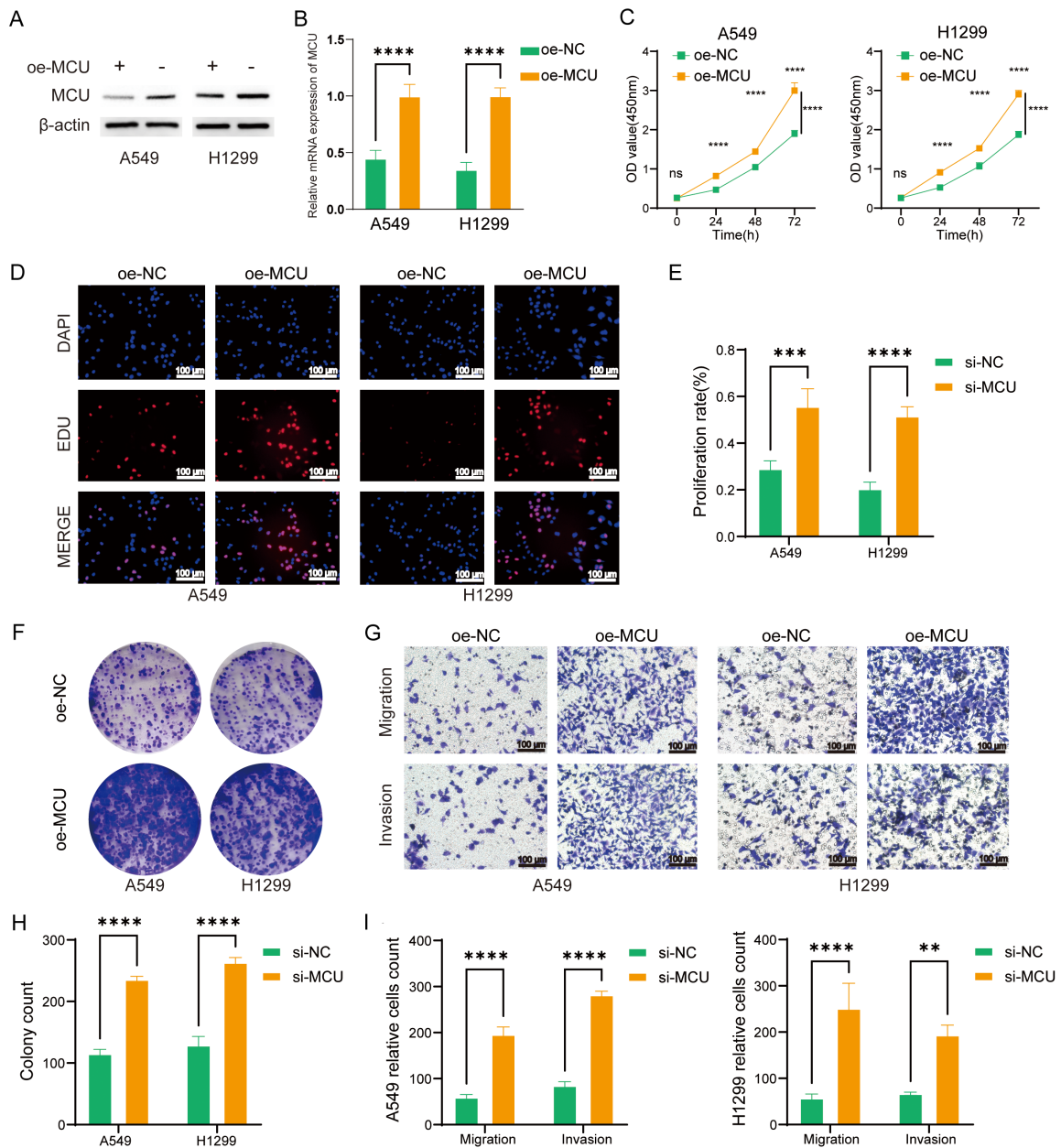


Figure 5. Overexpression of MCU enhances proliferation, colony formation, migration, and invasion in LUAD cell lines A549 and H1299. **A**, Western blot analysis of MCU protein expression in oe-MCU and oe-NC groups, with β -actin as internal control; **B**, qRT-PCR confirming increased MCU mRNA levels after overexpression; **C**, CCK-8 assay showing increased cell viability at 24, 48, and 72 hours in oe-MCU group; **D, E**, EdU assays illustrating enhanced proliferation following MCU overexpression, with fluorescence images and quantification; **F, G**, Colony formation assays indicating increased clonogenic ability in oe-MCU group; **H, I**, Transwell assays showing enhanced migration and invasion capacity in A549 and H1299 cells after MCU overexpression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns: not significant.

pressing MCU reduced early apoptosis in A549 and H1299 cells (**Figure 6B**). Cell cycle analysis showed that MCU knockdown resulted in G1 phase arrest of H1975 and PC9, and MCU overexpression decreased the G1 population in A549 and H1299 cells. On the other hand,

G2-phase populations were reduced after MCU knockdown and increased after MCU overexpression (**Figure 6C-F**).

Western Blotting has also been performed. Knockdown of MCU increased the expression

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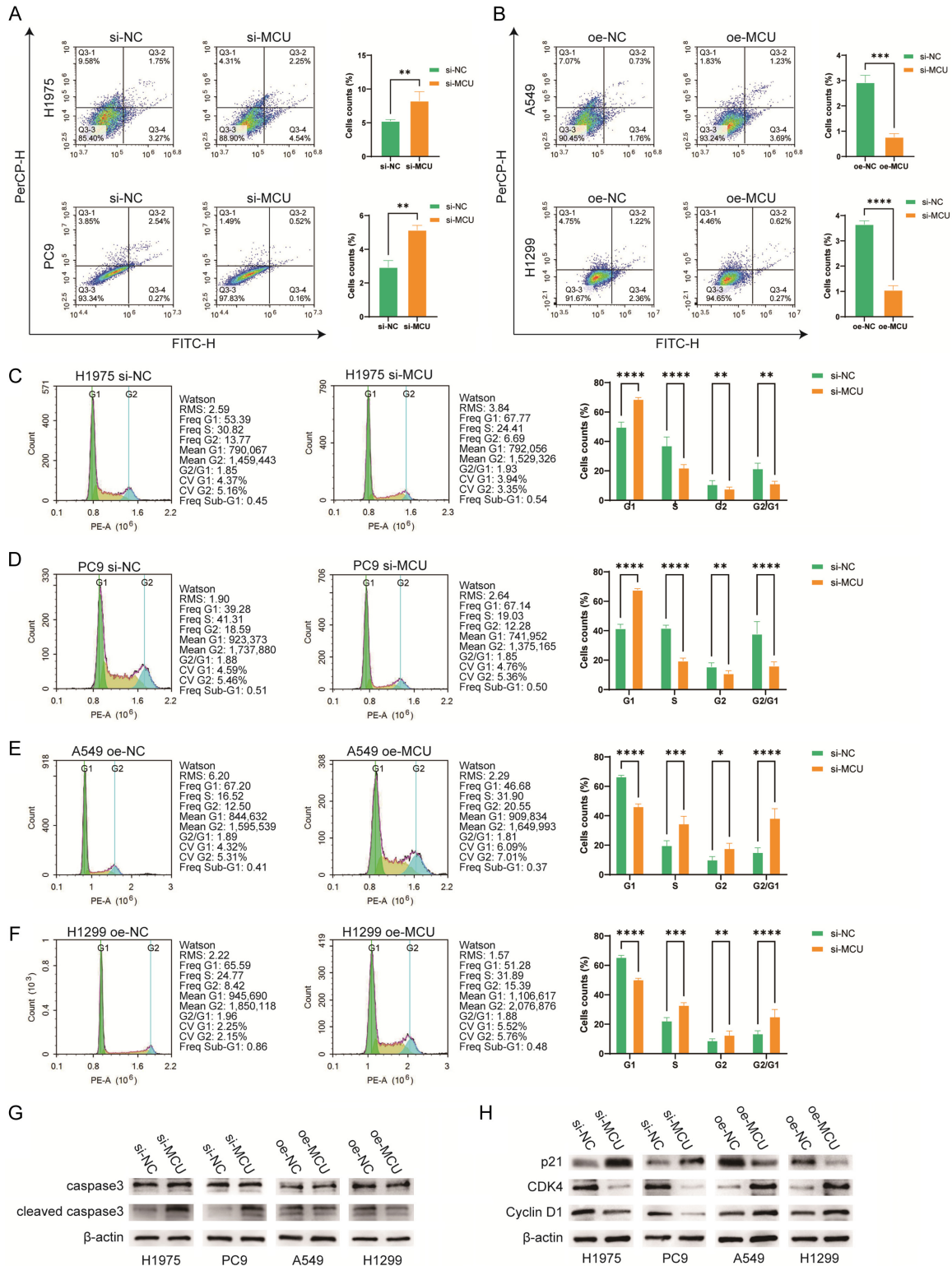


Figure 6. MCU regulates apoptosis and cell cycle progression in LUAD cells. A. Apoptosis analysis in H1975 and PC9 cells after MCU knockdown; B. Apoptosis levels in A549 and H1299 cells after MCU overexpression; C, D. Cell cycle phase distribution after MCU knockdown in H1975 and PC9; E, F. Cell cycle phase distribution after MCU overexpression in A549 and H1299; G. Western blot showing expression of cleaved caspase-3 after MCU knockdown and overexpression; H. Western blot analysis of cell cycle regulators (CDK4, Cyclin D1, and p21) in MCU knockdown and overexpression conditions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

of cleaved caspase-3 and thus promoted apoptosis (**Figure 6G** and [Supplementary Figure 2D, 2E](#)). Higher values were at the other end. Additionally, MCU suppression downregulated key cell cycle regulators such as CDK4 and cyclin D1, and significantly increased the cyclin-dependent kinase inhibitor p21. Overexpression of MCU reversed these changes (**Figure 6H** and [Supplementary Figure 2F-H](#)).

MCU mediates mitophagy and preserves mitochondrial function

MCU is a representative channel protein in the mitochondrion that regulates the balance of its internal environment; therefore, this study also examined whether MCU is involved in regulating various functional programmes of the mitochondrion. GSEA showed that MCU was closely associated with mitophagy-related biological processes (**Figure 7A**), and thus may be involved in the regulation of mitochondrial quality control.

Based on the above results, the expression levels of some key mitophagy-related genes were investigated following MCU inhibition. Western blot analysis showed that silencing MCU reduced the protein levels of PINK1 and PRKN in whole-cell lysates. Consistent with the above, the expression of LC3B in the mitochondrial fraction was also reduced after MCU depletion (**Figure 7B** and [Supplementary Figure 2I-K](#)), suggesting an impaired recruitment of autophagy-related factors to the mitochondria. Based on the above results, MCU knockdown reduces PINK1/PRKN-mediated mitophagy.

LC3B was then co-localised with mitochondria by immunofluorescence staining. Compared with control cells, MCU-deficient cells showed a reduced overlap between LC3B signals and mitochondria; further supported by quantitative co-localization analysis (**Figure 7C, 7D**), this was confirmed. The above results indicate that MCU deficiency suppresses mitophagy.

Since defective mitophagy often results in mitochondrial damage, we next observed a loss of mitochondrial membrane potential with JC-1 staining. Flow cytometry analysis showed that MCU knockdown resulted in a significant decrease in mitochondrial membrane potential (**Figure 7E, 7F**). Intracellular ROS were also measured by DCFH-DA staining. Knockdown of

the MCU significantly increased ROS in the control group (**Figure 7G, 7H**). Collectively, these results show that MCU depletion suppresses mitophagy and exacerbates mitochondrial dysfunction; that is, there is a decrease in mitochondrial recruitment of LC3B, loss of mitochondrial membrane potential, and increased ROS production.

Discussion

Recently, some research has started to study the molecular mechanisms of MCU. The application field of MCU in research on NSCLC is not fully developed. In this paper, we have systematically investigated the role of the MCU in regulating the immune microenvironment, cell proliferation and migration and invasion, cell cycle regulation, apoptosis, and mitophagy of NSCLC. Mitophagy plays a key role in regulating mitochondrial quality control and inhibiting the proliferation of tumor cells; therefore, based on this study, MCU may promote LUAD progression by maintaining mitophagy-dependent mitochondrial homeostasis. Collectively, the above results indicate that MCU has good prospects as a new prognostic marker for evaluating the progression of LUAD.

Immunoregulatory studies of MCU are still lacking. Previous studies have indicated that the activation and function of T cells are promoted by Ca^{2+} signaling [15]. T cell receptor (TCR) activation enhances mCa^{2+} uptake via MCU, thereby promoting T cell activation, ATP production, migration, and cytokine secretion [46, 47]. MCU inhibition inhibits the immune response and reduces the severity of autoimmune encephalomyelitis in model organisms [48]. MCU was concentrated in CD8^{+} T cells in breast cancer, and patients with high levels of MCU displayed increased tumor growth and greater intratumoral heterogeneity compared with those having low levels. Notably, patients in the high-MCU group had weaker immune responses and more immunosuppression [15, 47]. Other studies have also found that dendritic cell function, such as antigen presentation and cytokine secretion, is regulated by ion channel activity, which in turn may be altered by MCU-mediated Ca^{2+} signaling [46, 49].

Based on previous mechanistic studies [49], MARS2 is a protein located at the inner mitochondrial membrane that interacts with MCU

MCU as a biomarker in lung adenocarcinoma

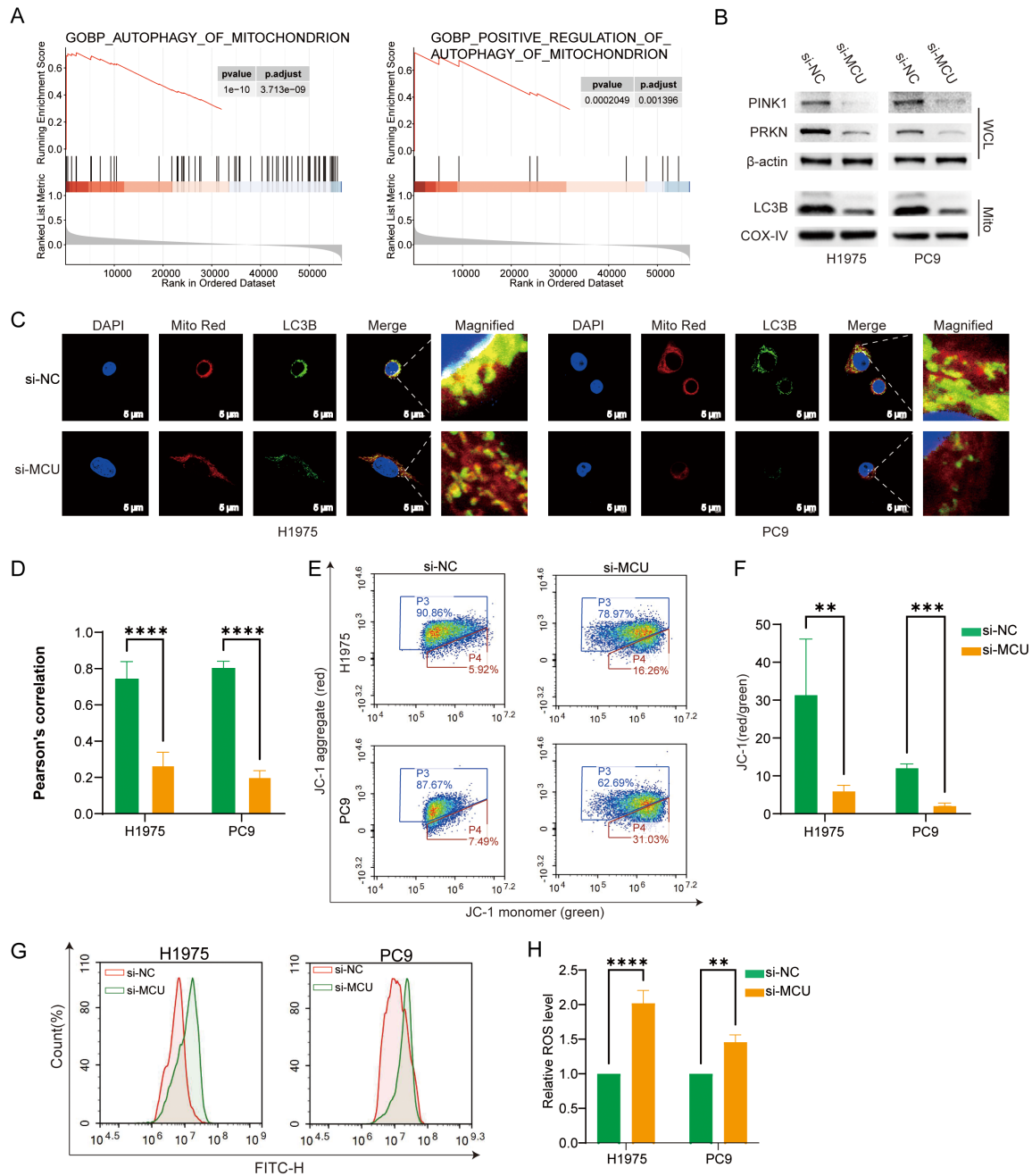


Figure 7. MCU regulates mitophagy and mitochondrial function. A. GSEA analysis showed that MCU-associated genes were significantly enriched in the “autophagy of mitochondrion” and “positive regulation of autophagy of mitochondrion” pathways; B. Western blot analysis was performed to detect the expression of mitophagy-related proteins, including PINK1, PRKN, and LC3B, in H1975 and PC9 cells; C, D. Immunofluorescence analysis was used to examine the colocalization of mitochondria and LC3B in H1975 and PC9 cells; E, F. JC-1 staining combined with flow cytometry was performed to assess changes in mitochondrial membrane potential in H1975 and PC9 cells; G, H. Flow cytometry was used to detect intracellular ROS levels in H1975 and PC9 cells. Data are presented as the mean $\pm$ SD. Statistical analysis was performed using two-sided t-tests. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

and regulates the function of MCU. MARS2-MCU is a molecular switch that controls the CaMKII/CREB signalling pathway to regulate p53 transcriptional activity and switch between

glycolysis and the pentose phosphate pathway (PPP). Madreiter-Sokolowski and others have shown in a study that altered MICU1 expression also reduces the proliferation and metastasis

of LUAD cells by interfering with Ca^{2+} entry [50]. Based on the above evidence, MCU-mediated mitochondrial Ca^{2+} dysregulation in solid tumors is shown to have a dual function [51, 52]. It can boost mitochondrial metabolism or trigger mitochondrial dysfunction and oxidative stress and apoptosis through the generation of excessive Ca^{2+} . Therefore, many solid tumors are highly dependent on MCU function [53]. Modulation of mitochondrial metabolism, cell cycle regulation and redox homeostasis by MCU all promote the proliferation and survival of tumor cells; therefore, MCU is now a prospect for LUAD treatment.

First, we show that the MCU is significantly overexpressed in the tumor tissue compared with adjacent normal tissue of NSCLC. Function enrichment analysis indicates that the genes involved in the MCU belong to the pathways of immune response, apoptosis and the cell cycle. Stratification of patients according to MCU expression showed that those in the high-expression group had significantly lower immune scores, checkpoint gene expression and overall immune pathway activity. TIDE scores also show that most of the patients with high-MCU are immunocompromised and less likely to respond to immunotherapy.

To further verify the biological function of MCU, four cell lines were selected for experiment: H1975 and PC9 (high MCU expression), and A549 and H1299 (low expression). MCU knock-down in H1975 and PC9 cells significantly inhibited cell proliferation, migration, and invasion. Conversely, MCU overexpression in A549 and H1299 cells significantly promoted the malignant behavior of these cells; next, apoptosis and cell-cycle pathways involved in enrichment analyses were investigated. Knockdown of MCU increased cleaved caspase-3 levels and thus promoted apoptosis; overexpressed MCU, however, suppressed the expression of cleaved caspase-3. Overexpression of MCU inhibited apoptosis and promoted the division and spread of cancer cells, as shown by flow cytometry. MCU knockdown significantly increased the expression of p21, a cell cycle inhibitor, and reduced the expression of CDK4 and Cyclin D1 [54]. Therefore, MCU deficiency induces G1-phase arrest by interfering with the formation of the CDK4/Cyclin D1 complex and inhibits the G1-S phase transition, leading to

reduced growth and migration of cancer cells [55].

GSEA in this study shows that MCU expression was significantly associated with mitophagy-related pathways. MCU inhibition causes mitochondrial membrane depolarization and an increase in intracellular ROS. MCU is a typical channel for mitochondrial calcium entry that regulates both energy supply and calcium homeostasis in the mitochondrion. Disruption of the MCU function can harm the mitochondria and reduce metabolic activity; as a result, there will be oxidative stress and activation of mitochondrial quality control. MCU knock-down reduced PINK1 and PRKN expression, decreased mitochondrial LC3B accumulation, and impaired mitophagy. These findings suggest that MCU promotes LUAD progression by maintaining mitophagy and mitochondrial function.

Based on the above results, the direction of research is now focused on targeting MCU-dependent mitochondrial calcium signalling to disturb mitochondrial homeostasis and inhibit LUAD progression.

Conclusion

In short, our study shows that MCU is highly expressed in LUAD and promotes the growth of tumors. Integrated bioinformatics analysis and experiments have shown that MCU promotes the proliferation, migration and invasion of cells; it inhibits apoptosis and accelerates the cell cycle. A high expression of the MCU was associated with less immune infiltration and immune evasion; it also reduced the effect of immunotherapy. Mitochondrial depletion results in mitochondrial membrane depolarization and thus ROS generation; therefore, PINK1/Parkin-mediated mitophagy is induced to restore mitochondrial homeostasis. Collectively, the above studies show that MCU regulates immune remodelling, mitochondrial function and malignant progression in lung adenocarcinoma (LUAD), and thus may be used as prognostic indicators or targets for new drugs.

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Disclosure of conflict of interest

None.

Address correspondence to: Haoling Zhang, Department of Biomedical Sciences, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Kepala Batas 13200, Penang, Malaysia. E-mail: zhanghaolingedu@163.com; Honglin Zhao and Jun Chen, Department of Lung Cancer Surgery, Tianjin Medical University General Hospital, Tianjin 300052, The People's Republic of China. E-mail: navyzhao@aliyun.com (HLZ); huntercj2004@qq.com (JC); Xiaobin Shang and Yin Li, Department of Thoracic Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100000, The People's Republic of China. E-mail: shangxiaobin626@live.cn (XBS); liyin_thorax@163.com (YL)

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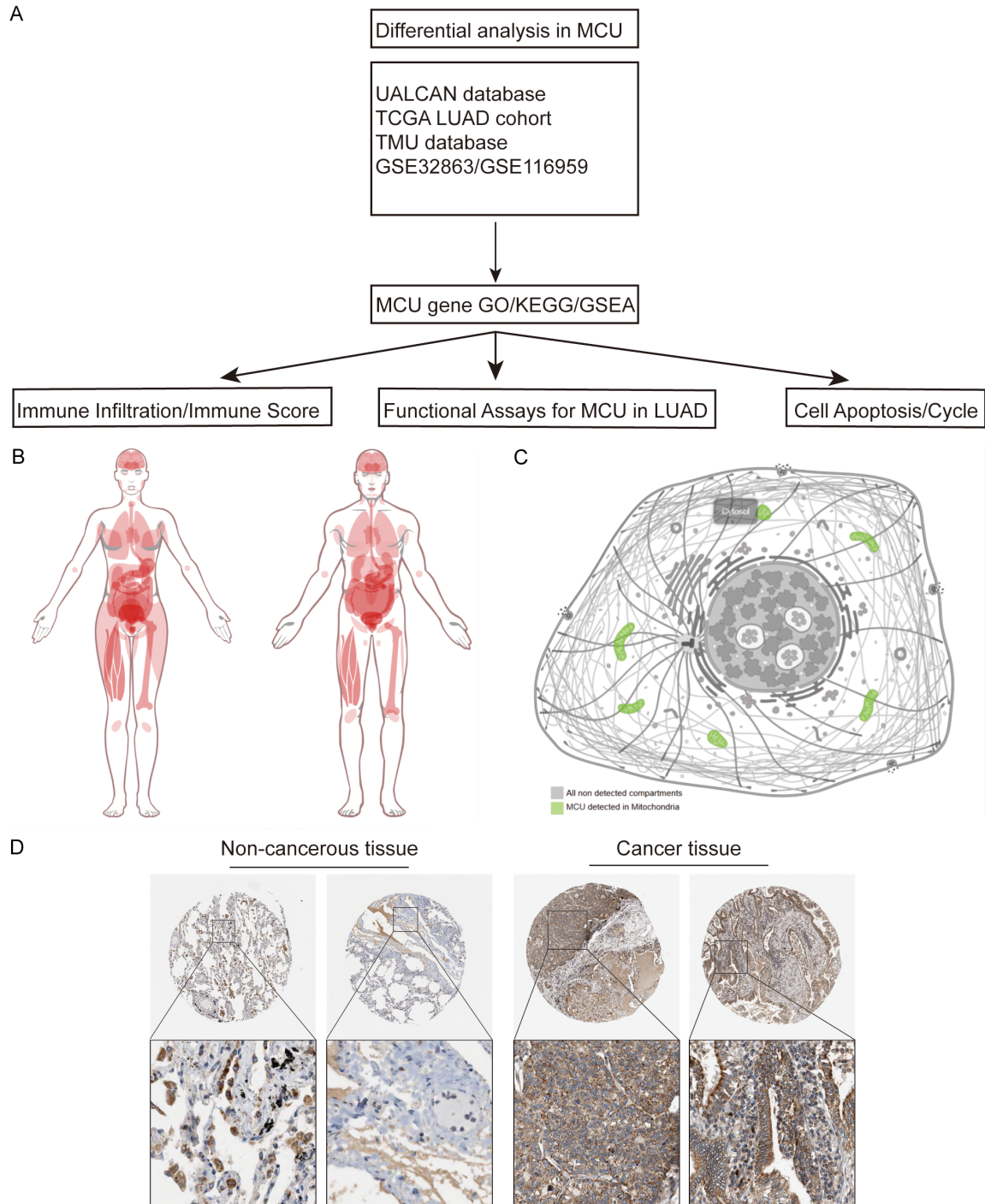
Supplementary Table 1. List of primer sequences used for qRT-PCR analysis in this study

		Sequence (5'-3')
β-actin	Sense	CATGTACGTTGCTATCCAGGC
	Antisense	CTCCTTAATGTCACGCACGAT
si-MCU	Sense	GUACGAAUUGAGAUUAGCATT
	Antisense	UGCUAUCUCAAUUCGUACTT

Supplementary Table 2. Primary and secondary antibodies used for Western blotting, including suppliers and dilution ratios

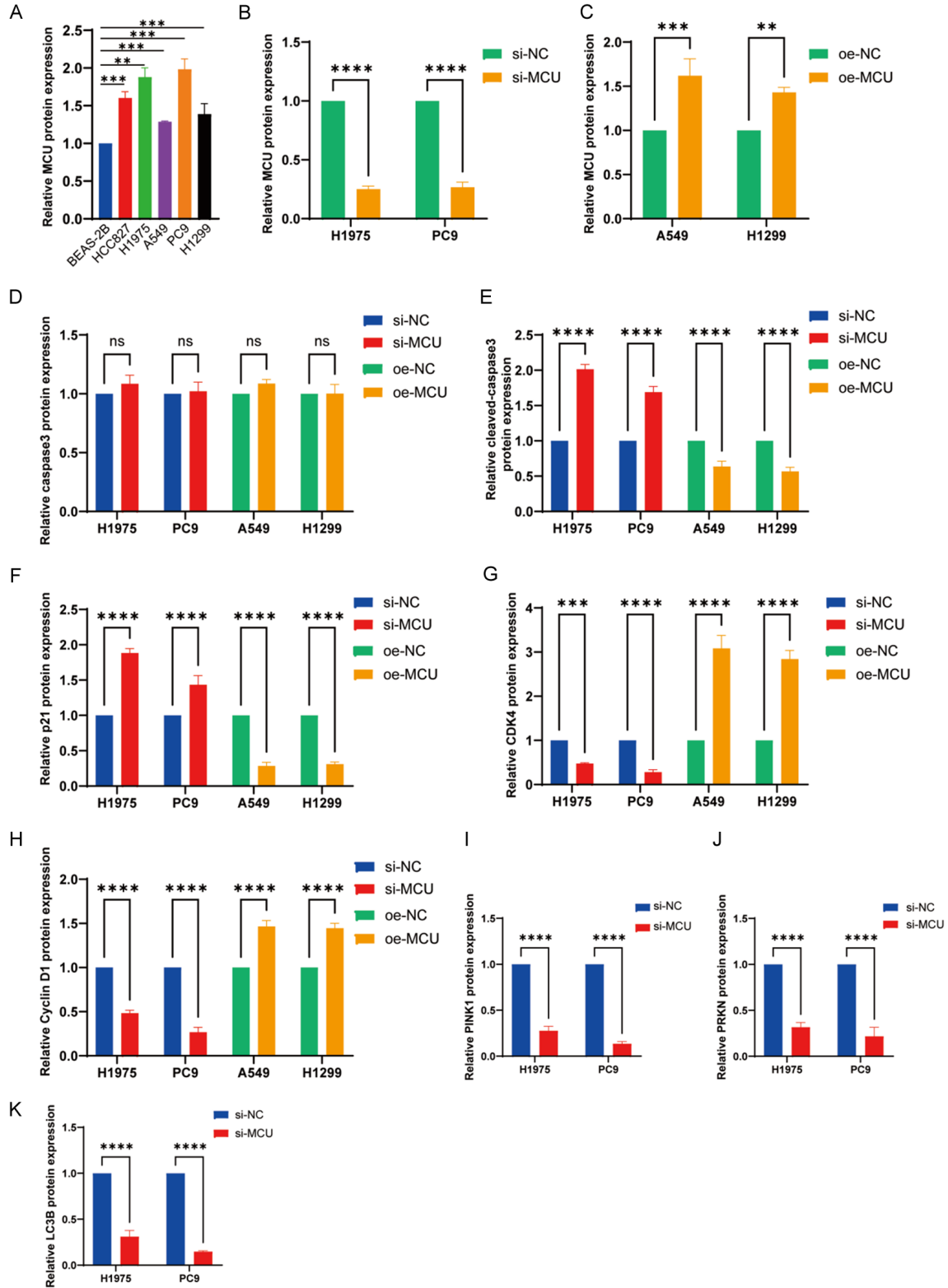
Antibody	Manufacturer	Western blot concentration	Secondary antibody
β-actin	Servicebio	1:5000	Rabbit (1:5000)
MCU	Servicebio	1:500	Rabbit (1:5000)
caspase 3	Proteintech	1:1000	Rabbit (1:5000)
cleaved caspase 3	Proteintech	1:1000	Rabbit (1:5000)
p21	Proteintech	1:2000	Rabbit (1:5000)
CDK4	SANTA	1:1000	Rabbit (1:5000)
Cyclin D1	Proteintech	1:5000	Rabbit (1:5000)
PINK1	Proteintech	1:2000	Rabbit (1:5000)
PRKN	Proteintech	1:4000	Rabbit (1:5000)
LC3B	Proteintech	1:2000	Rabbit (1:5000)
COX-IV	Proteintech	1:10000	Rabbit (1:5000)

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Supplementary Figure 1. Workflow and MCU expression distribution. A. Overall workflow diagram; B. Spatial distribution of MCU in vivo and in vitro; C. Immunohistochemical staining of MCU in tumor and normal tissues based on the HPA database. D. IHC staining of MCU protein expression based on the HPA database.

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Supplementary Figure 2. Quantitative analysis of Western blot data. A. Basal expression levels of MCU in different cell lines; B, C. Western blot analysis and corresponding densitometric quantification of MCU expression following knockdown in H1975 and PC9 cells, and overexpression in A549 and H1299 cells; D, E. Western blot analysis and quantitative analysis of apoptosis-related proteins caspase-3 and cleaved caspase-3 in four cell lines after MCU knockdown or overexpression; F-H. Western blot analysis and quantitative analysis of cell cycle-related proteins p21,

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CDK4, and Cyclin D1 in four cell lines following MCU knockdown or overexpression; I-K. Western blot analysis and corresponding densitometric quantification of the mitophagy-related proteins PINK1, PRKN, and LC3B in H1975 and PC9 cells following MCU knockdown. β -actin was used as a loading control. Data are presented as the mean $\pm$ standard deviation (SD) from at least three independent experiments. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.