

KMO downregulation promotes hepatocellular carcinoma growth via 3-HAA-mediated mitochondrial mass and function imbalances

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Abstract. Kynurenine (Kyn) 3-monooxygenase (KMO) is a key enzyme of the tryptophan (Try)-Kyn pathway and is located on the outer membrane of mitochondria. Notably, it has not yet been elucidated as to whether KMO is involved in hepatocellular carcinoma (HCC) progression by affecting mitochondria. In the present study, KMO was revealed to be downregulated in HCC patients and this downregulation was associated with a poor prognosis. Notably, the downregulation of KMO promoted the proliferation and migration of HCC

cells and increased mitochondrial mass. The levels of the Try metabolite 3-hydroxyanthranilic acid (3-HAA) were elevated in HCC cells overexpressing KMO. The results indicated that 3-HAA may inhibit HCC cell growth promoted by KMO downregulation and reverse the KMO downregulation-induced increase in mitochondrial mass. Furthermore, KMO and 3-HAA were shown to regulate the expression of the transcription factor nuclear receptor subfamily 4 group A member 1 (NR4A1) and reduce NR4A1 mitochondrial translocation, thus inhibiting the growth of HCC cells. In summary, the current study elucidated that low KMO expression in HCC affects mitochondrial mass and function by reducing the level of the Try metabolite 3-HAA, downregulating the expression of NR4A1 and promoting its mitochondrial translocation, which in turn may promote the progression of HCC. These findings provide new insights into the treatment of HCC, potentially targeting the mitochondria and the Try-Kyn pathway.

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Abbreviations: HCC, hepatocellular carcinoma; KMO, kynurenine 3-monooxygenase; 3-HAA, 3-hydroxyanthranilic acid; NR4A1, nuclear receptor subfamily 4 group A member 1; Try, tryptophan; Kyn, kynurenine; TDO, tryptophan-2,3-dioxygenase; mtDNA, mitochondrial DNA; GEO, gene Expression Omnibus; DEGs, differentially expressed genes; MRDEGs, mitochondrial related DEGs; TCGA, the Cancer Genome Atlas; LIHC, liver hepatocellular carcinoma. WGCNA, weighted gene co-expression network analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction

Key words: hepatocellular carcinoma, kynurenine 3-monooxygenase, 3-hydroxyanthranilic acid, nuclear receptor subfamily 4 group A member 1, mitochondrial mass

Introduction

According to 2022 cancer statistics, among 36 tumors, the global incidence and mortality rates of liver cancer rank sixth and fourth, respectively (1). Hepatocellular carcinoma (HCC) is the primary histological subtype of primary liver cancer, accounting for 75-85% of cases (2,3). Due to the lack of individualized diagnostic markers, HCC is often diagnosed late, which is the main reason for its poor prognosis (4). The treatment bottlenecks of HCC mainly include tumor heterogeneity, drug resistance and optimization of multidisciplinary comprehensive treatment and biomarker indicators are essential to guide treatment selection (5). Mitochondria, as essential organelles in eukaryotic cells, play a central role in energy metabolism, maintaining redox balance and regulating cell apoptosis. Mitochondrial DNA (mtDNA) mutations, electron transport chain defects, oxidative stress, mitochondrial autophagy and other types of mitochondrial dysfunction are

closely related to the occurrence of tumors (6). In addition, strategies that target mitochondria serve an important role in tumor treatment (7).

Kynurenine (Kyn) 3-monooxygenase (KMO) is one of the three rate-limiting enzymes [indoleamine-2,3-dioxygenase (IDO), tryptophan (Try)-2,3-dioxygenase (TDO) and KMO] of the Try-Kyn pathway and is located on the outer membrane of mitochondria (8,9). However, studies regarding the role of KMO in mitochondrial structural and function are insufficient. Maddison *et al* (10) reported that deficiency in the KMO ortholog (cinnabar) in *Drosophila* triggered morphological elongation and a reduction in respiratory capacity in mitochondria. At present, it is widely known that mitochondrial dynamics are involved in a range of diseases, including cancer (11). Dysregulated mitochondrial dynamics can promote cancer development by affecting tumor cell proliferation, metastasis, drug resistance and the tumor microenvironment, which highlights the potential of targeting mitochondrial dynamics as a promising anticancer strategy (11).

KMO has a dual effect on tumor progression in various types of cancer, influencing tumor occurrence, development and immune escape. KMO is highly expressed in breast cancer and colon cancer and is associated with poor prognosis, whereas its expression is downregulated in clear cell and chromophobe cell renal cell carcinoma, as well as in endometrial carcinoma and nasopharyngeal carcinoma (12–16). These studies suggest the complexity of the function of KMO in tumors and the research on KMO in tumor progression is in its infancy (17). As research on Try metabolism in tumors advances, targeting key enzymes such as IDO1 and TDO to disrupt immunosuppressive signaling has emerged as a promising strategy. However, the inconsistent outcomes of IDO1 inhibitors in advanced trials underscore the necessity for further investigation into Try metabolism within cancer contexts (18). In HCC, Jin *et al* (19) reported that KMO expression was higher in cancer tissues than in adjacent tissues and its expression in liver cancer cells promoted the malignant progression of tumor cells. However, Shi *et al* (20) revealed that KMO and the Kyn derivative 3-hydroxyanthranilic acid (3-HAA), also the downstream product of KMO, may inhibit tumor formation by inducing cell apoptosis via activating the transcription factor Yin Yang 1 (YY1). Notably, there is a lack of research on whether KMO located on the mitochondria promotes the progression of HCC by affecting mitochondrial homeostasis and whether the effect of KMO-induced mitochondrial disorder is related to Try metabolism.

The present study systematically investigated the role of KMO-mediated mitochondrial dysfunction in the malignant progression of HCC using public data, clinical samples and HCC cell lines. The differential expression of genes related to mitochondria, including KMO, were initially screened across multiple public HCC datasets. Subsequently, the expression of KMO was validated in clinical samples of HCC. Furthermore, by interfering with KMO expression in HCC cells, changes in mitochondrial mass and function were observed. The current study also explored the impact of KMO-mediated downstream 3-hydroxyanthranilic acid (3-HAA) on mitochondrial mass and function, aiming to clarify the potential mechanisms underlying KMO-mediated mitochondrial dysfunction in HCC and to provide new strategies for HCC treatment.

Materials and methods

Patients and specimens. A total of 31 pairs of tissue specimens, including tumor and adjacent tissues (distance from tumor edge >1 cm), from 31 patients with pathologically confirmed HCC were collected from the Department of Oncology and Vascular Intervention, First Hospital of Shanxi Medical University between January 2021 and December 2022. The patients (29 men and 2 women) ranged in age from 50–78 years. The clinicopathological features of these patients with HCC is shown in Table SI. This study was approved by the ethics committee of the First Hospital of Shanxi Medical University (approval number: 2021SLL086). All patients provided written informed consent. All research was conducted in strict accordance with the Helsinki Declaration and relevant institutional and national regulations.

Public database. The HCC-related GSE101728, GSE45050, GSE84598 and GSE121248 datasets from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/gds/?term=>) were used for screening of shared differentially expressed genes (DEGs). The Cancer Genome Atlas (TCGA)-liver hepatocellular carcinoma (LIHC) dataset containing the clinical information and mRNA expression data of 371 HCC patients and 50 normal controls was downloaded from the TCGA database (<https://portal.gdc.cancer.gov/>). The somatic mutation profiles of HCC patients were obtained from the University of California, Santa Cruz (UCSC Xena Browser, <https://xenabrowser.net/datapages/>). HCC related single cell sequencing dataset GSE202642 came from the GEO database.

DEGs screening. DEGs from the HCC-related GSE101728, GSE45050, GSE84598 and GSE121248 datasets were screened using the GEO 2R analysis tool (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>). The ‘limma’ R package (version 3.52.0; <https://bioinf.wehi.edu.au/limma/>) was used to normalize the TCGA-LIHC dataset and identify DEGs. Thresholds were set as $P < 0.05$ and $|\log FC| > 1$.

Mitochondrial-related DEGs (MRDEGs) identification. The list of mitochondrial-related genes was obtained from MitoCarta3.0 (<https://www.broadinstitute.org/mitocarta/mitocarta30-inventory-mammalian-mitochondrial-proteins-and-pathways>), which includes 1,136 human mitochondrial-related genes. Mitochondrial-related genes were intersected with the DEGs from the four HCC-related GEO datasets to obtain MRDEGs.

Analysis of single cell sequencing data. The GSE202642 dataset comprising seven HCC samples and 4 adjacent non-tumor samples was analyzed, it contained a total of 115,732 cells and 36,601 genes. The ‘Seurat’ R package (version 4.3.0; <https://github.com/satijalab/seurat>) was used to process single cell sequencing data as follows: i) ‘DoubletFinder’ was applied to remove doublets; ii) the ‘PercentageFeatureSet’ function calculated mitochondria and red blood cells content per cell. Quality control was performed using the criteria: i) Detected genes per cell >500; ii) detected genes per cell <6,000; iii) mitochondrial genes <20; iv) red blood cells <1. ‘NormalizeData’ and ‘ScaleData’ functions were then used to normalize and

scale the data. 'RunPCA' function performed linear dimensionality reduction with $\text{dim}=20$. 'FindClusters' function clustered cells with $\text{resolution}=0.9$. 'RunTSNE' function performed tSNE dimensionality reduction. 'FindAllMarkers' function identified DEGs in each cell cluster. The 'SingleR' R package (version 2.0.0; <https://github.com/SingleR-inc/SingleR>) was used to identify and annotate cell types. KMO expression levels in normal and HCC samples were compared across different cell types.

Survival analysis. Overall Survival analysis of genes in HCC patients was performed using the Kaplan-Meier Plotter online tool (<http://kmplot.com/analysis/>).

Weighted gene co-expression network analysis (WGCNA). WGCNA was constructed using the DEGs matrix of 340 HCC samples via the 'WGCNA' R package (version 1.71; <https://CRAN.R-project.org/package=WGCNA>). Sample clustering was performed using the 'hcluster' function to identify and remove outliers. The optimal soft threshold power β for network construction was determined using the 'pickSoftThreshold' function. The adjacency degree matrix was transformed into a topological overlap matrix (TOM) using 'TOMsimilarity'. Gene expression similarity was then calculated and the minimum value of module genes was set to 30 genes. According to the similarity, genes were divided into different co-expression modules and the module allocation diagram under gene tree diagram was drawn by DynamicTreeCut. HCC samples were divided into high and low KMO expression groups based on the median value of KMO expression. Correlation analysis was performed between gene co-expression module and KMO expression status. Pearson's correlation coefficient and P-value were calculated using the 'cor' function. The co-expression module with the largest correlation coefficient $P<0.05$ was regarded as the key functional module and the genes within this module were considered KMO-co-expressed hub genes.

Gene enrichment analysis. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed using clusterProfiler (version 4.8.0; <https://yulab-smu.top/contribution-knowledge-mining/>) and ggplot2 (version 3.4.1; <https://CRAN.R-project.org/package=ggplot2>) packages in R.

Protein-protein interaction (PPI) and correlation analysis. To investigate the relationship between KMO and Try metabolic pathway genes, Try metabolic pathway genes were downloaded from the GSEA official website (<https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>) and analyzed using the STRING database (<https://string-db.org/>). The PPI network was visualized using Cytoscape 3.9.0 software (<https://cytoscape.org/>). The hub genes related to KMO were captured using CytoHubba of Cytoscape. Correlation heatmaps and lollipop plots were generated using the online SRplot tool (<http://www.bioinformatics.com.cn/SRplot>) (21).

Cell culture and KMO expression cell model. The HCC cell lines MHCC-97H and HCCLM3 (Cell Resource Center, Institute of Basic Medicine, Chinese Academy of Medical

Sciences) were cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO₂.

KMO-overexpressing lentivirus were constructed by Shanghai GenePharma Co., Ltd. using the 3rd lentiviral, LV5 (EF-1a/GFP&Puro). 293T cells (Cell Bank of Chinese Academy of Sciences) was selected to package the lentivirus using shuttle plasmid H6370 and packaging plasmid (pGag/Pol, pRev, pVSV-G). The lentivirus titer of 293T cells after 72 h of infection with the collected lentivirus stock solution was 9×10^8 TU/ml. Multiplicity of infection (MOI) was calculated according to virus titer and the optimal MOI (1 number/cell) was determined for HCC cell infection by pre-experiment. For lentiviral overexpression, cells were seeded in 24-well plates at 5×10^4 cells/well. When confluence reached 40-60%, cells were infected with KMO-overexpressing lentivirus at 1×10^5 TU/ml. After 24 h, the medium was replaced with fresh culture medium. Upon reaching 70-80% confluence, positive cells were screened using 1 $\mu\text{g/ml}$ puromycin concentration (the optimal concentration determined by puromycin concentration gradient experiment), and 0.5 $\mu\text{g/ml}$ was the maintenance concentration of cultured cells. For short interfering (si)RNA knockdown, siRNA targeting KMO (5'-GGAGCCTATTCAACTGTCA-3', 5'-GCGAGCACATGTCAACTCA-3' and 5'-CCAAGGTATTCCCATGAGA-3') and control siRNA was designed and synthesized by Guangzhou RiboBio Co., Ltd. Cells were seeded in 6-well plates at 3×10^5 cells per well and transfected at 70-80% confluence using Lipo8000 reagent (Beyotime Institute of Biotechnology) with the screened siRNA sequence (5'-CCAAGGTATTCCCATGAGA-3') having the highest knockdown efficiency and a control siRNA. Overexpression and knockdown efficiencies were assessed using qPCR and western blotting. KMO stably overexpressing cell lines and transiently transfected KMO-knockdown cells (harvested at 48 h) were used for subsequent studies.

Immunofluorescence. Cells were cultured in confocal dishes (1,000 cells/dish) for 24 h, then stained with Mito-tracker at 37°C for 25 min in the dark. After washing with ice-cold PBS, cells were fixed with 4% paraformaldehyde at 37°C for 1 h, permeabilized with 0.2% TritonX-100 for 10 min, after TBST wash buffer (0.05% Tween 20) washing 3 times (5 min/time), cells were blocked with 2% BSA (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min. Cells were incubated with 1:100 diluted KMO or NR4A1 primary antibody (cat no. 10698-1-AP and 12235-1-AP, Proteintech Group, Inc.) overnight at 4°C, followed by fluorescent secondary antibody (1:200; cat no. SA00013-2; Proteintech Group, Inc.) at room temperature for 1 h. After DAPI staining (1:200) at room temperature for 15 min, cells were imaged at a magnification of x100.

Immunohistochemical staining. Paraffin-embedded sections (thickness: 5 μm) were dewaxed, cleaned with PBS and treated with 3% H₂O₂ to block endogenous peroxidase at room temperature for 10 min. Antigen retrieval was performed using sodium citrate buffer, followed by PBS washing. Sections were blocked with 5% BSA at 37°C for 30 min, then incubated with 1:100 diluted KMO primary antibody (cat no. 10698-1-AP, Proteintech Group, Inc.) overnight at 4°C. After washing, sections were incubated with 1:1,000 diluted HRP-conjugated secondary antibody (cat no. KIT-5010;

Maxim Biomedical, Inc.) for 30 min at room temperature. DAB staining was performed for 1 min, followed by hematoxylin counterstaining for 5 min. Sections were dehydrated, mounted and imaged using a light microscope. The staining intensity (SI) and percentage of positive cells (PP) were used to calculate the IHC score (22). SI was graded as follows: 0 for negative, 1 for low positive, 2 for moderate positive, and 3 for high positive. PP was classified into four categories: 0 (0-9% positive cells), 1 (10-25%), 2 (26-50%), 3 (50-75%), and 4 (76-100%). The final immunohistochemical score (IS) was derived by multiplying SI and PP (IS=SI x PP). Quantitative analysis was performed using ImageJ software (version 1.53a, National Institutes of Health).

mRNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from tissue samples or cultured cells using RNAiso Plus (Takara Bio, Inc.). RNA was reverse-transcribed into cDNA using the PrimeScript RT Master Mix Reverse Transcription Kit (Takara Bio, Inc.). The target gene expression was quantified by two-step qPCR using TB Green® Premix Ex Taq II (Takara Bio, Inc.) with the procedure: pre-denaturation at 95°C for 30 sec, 1 cycle; denatured at 95°C for 5 sec, annealed at 60°C and extended for 30 sec, 40 cycles. All operations of RNA extraction, cDNA synthesis, and qPCR were carried out according to the manufacturer's protocols. Relative expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method according to previous study (23). Primer sequences are listed in Table I. Each sample was analyzed in triplicate in three independent experiments.

Protein extraction and western blotting. Cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology), incubated on ice for 30 min and centrifuged at 12,000 x g for 20 min at 4°C. The supernatant was collected and protein concentration was measured using a BCA kit (Beyotime Institute of Biotechnology) at 562 nm.

Equal amounts of protein ($\geq 20 \mu\text{g}$) were separated by 10% SDS-PAGE and subsequently transferred to the PVDF membrane at 100 V for 1 h. Membranes were blocked with 5% non-fat milk for 1 h at room temperature. The membrane was incubated with 1:500 diluted KMO (cat no. 10698-1-AP; Proteintech Group, Inc.) or 1:10,000 diluted β -Actin (cat no. 66009-1-Ig; Proteintech Group, Inc.) primary antibodies overnight at 4°C. After washing, membranes were incubated with 1:10,000 diluted horseradish peroxidase-conjugated secondary antibody (cat no. SA00001-2; Proteintech Group, Inc.) for 1 h at room temperature. Finally, the chemiluminescent substrate (1:1 ratio of solutions A and B) was added to the PVDF membrane for imaging after washing. Relative protein expression was calculated using ImageJ software (version 1.53a, National Institutes of Health).

CCK-8 assay. Cell viability was assessed using the CCK-8 assay kit (MeilunBio Co., Ltd.). Cells were seeded in 96-well plates at 3,000 cells per well. Upon cells reaching the logarithmic growth phase, siRNA transfected experiment was performed. For the 3-HAA group, the medium was replaced with one containing 100 μM 3-HAA (24). CCK-8 reagent was added at 0, 24, 48, 72 and 96 h post-transfection and incubated at 37°C for 2 h. Absorbance at 450 nm was measured

Table I. Primer sequences used in reverse transcription-quantitative PCR.

Gene	Primers (5' to 3')
KMO	Forward: CACTGTGTACTGCTGGGAGA Reverse: ATCGCGTGATCATCTGGGAT
β -actin	Forward: ACTCTTCCAGCCTTCCTTCC Reverse: TCTCCTTCTGCATCCTGTCTG
KYNU	Forward: TGCTGAGTTCATGGCCAAAC Reverse: AGTGATCCTGTTCAGTGGGG
ND1	Forward: CCCTAAACCCGCCACATCT Reverse: GAGCGATGGTGAGAGCTAAGGT
HGB	Forward: GTGCACCTGACTCCTGAGGAGA Reverse: CCTTGATACCAACCTGCCAG
MT-ATP6	Forward: ACAGTGATTATAGGCTTTCGCTC TAAG Reverse: ATTGGTTGAATGAGTAGGCTGA TGG
MT-CO1	Forward: GCAGGAACAGGTTGAACAGTC TAC Reverse: TAGGTGTAAGGAGAAGATGGTT AGGTC
SOX4	Forward: GCAAGATCATGGAGCAGTCG Reverse: CCGGACTTCACCTTCTTCTCT
HMGA1	Forward: GTAGGGAGTCAGAAGGAGCC Reverse: CCTCCTCTTCTCTCTTCTCC
NR4A1	Forward: AGAAGATCCCTGGCTTTGCT Reverse: CAGGGACATCGACAAGCAAG
FOXO1	Forward: CCGAGCTGCCAAGAAGAAAG Reverse: ATGCACATCCCCTTCTCCAA

using a multifunctional enzyme-linked immunosorbent assay instrument.

EdU staining experiment. Cell proliferation was assessed using the EdU assay kit (Guangzhou RiboBio Co., Ltd.). Cells were seeded in a 96-well plate at 3,000 cells per well and treated as described. After 24 h, cells were transferred to confocal dishes. After 48 h, 200 μl of 50 μM EdU was added and incubated at 37°C for 2 h in the dark. Cells were then treated sequentially with glycine (2 mg/ml, room temperature, 5 min), Triton X-100 (0.5%, room temperature, 10 min), Apollo (30 min, room temperature, in the dark) and Hoechst 33342 staining for 30 min (room temperature, in the dark).

Clonal formation assay. Cells were seeded in a 6-well plate at 500 cells per well. When visible colonies appeared after 10-14 days, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, then stained with 0.1% crystal violet at room temperature for 30 min. Colonies were defined as cell clusters containing ≥ 50 cells. Plates were rinsed, inverted and colonies were photographed and counted.

Scratch and transwell tests. The HCC cell lines MHCC-97H and HCCLM3 were cultured for 24 h to 80% confluence, then

scratched with a 200 μ l pipette tip. For siKMO groups, cells were transfected with siKMO RNA or siControl RNA (20 nM) and cultured at 37°C. After 6 h of transfection, the complete medium was replaced and the culture medium was replaced with fresh medium containing 1% FBS after 24 h of transfection. For KMO overexpression groups, the cells with stable overexpression of KMO and the EV control group were directly scratched after 24 h of culture and then cultured in 1% FBS medium. The wound closure was imaged at 0 and 48 h after scratch and ImageJ software (version 1.53a, National Institutes of Health) was used to quantify the migration distance. For other cells to be tested by Transwell, transfection was performed followed by 24 h of culture. A 24-well plate was prepared with 700 μ l of complete medium per well. 200 μ l of serum-starved cell suspension (KMO knockdown, KMO overexpression, or corresponding control cells) was added to each well and cultured for 48 h. Non-migrated cells on the upper layer of the chamber membrane were removed with a cotton swab and cells on the lower surface were fixed with methanol at room temperature for 10 min, then stained with crystal violet at room temperature for 20 min and counted under a light microscope.

Flow cytometry analysis of apoptosis. Following the instructions for the Annexin V-FITC apoptosis detection kit (Bestbio Technology Co., Ltd.), cells were washed twice with pre-cooled PBS to obtain cell pellets, then re-suspended in binding buffer. Cells were stained with 5 μ l Annexin V-FITC at 4°C for 15 min, followed by 10 μ l propidium iodide dye for 10 min at 4°C in the dark. Samples were immediately detected using flow cytometry (FACSCelesta; BD Biosciences). And the apoptotic rate of HCC cells (the sum of the percentages of early + late apoptotic cells) was analyzed using FlowJo 7.6 software (BD Biosciences).

MitoTracker staining. Mitochondrial biogenesis was detected by using MitoTracker staining method. Cells were seeded in confocal dishes at 1,000 cells/dish. At 48 h after siKMO transfection or 100 μ M 3-HAA treatment, cells were stained with MitoTracker (200 nM) for 25 min at 37°C in the dark. After washing twice with DMEM, cells were imaged by confocal microscopy at a magnification of x100. The total mitochondrial fluorescence intensity was quantified using ImageJ (version 1.53a, National Institutes of Health).

High performance liquid chromatography (HPLC) combined with mass spectrometry (MS). Targeted Try metabolism analysis was performed by Shanghai Zhongke New Life Biotechnology Co. Ltd.

Sample preparation. Cells were harvested, washed three times with PBS and scraped with 1 ml precooled methanol/acetonitrile/water (2:2:1, v/v). Samples were collected, labeled, sealed and stored at -80°C until analysis.

Chromatographic conditions. Samples were analyzed on an Agilent 1290 UHPLC system (Agilent Technologies, Inc.) with a 4°C autosampler and column oven at 40°C. Mobile phases: A (20 mM ammonium formate + 0.2% formic acid in water), B (0.2% formic acid in methanol). Flow rate: 400 μ l/min; injection volume: 5 μ l. Gradient elution: 0-2 min, 10% B; 2-9 min, 10 to 100% B; 9-11 min, 100% B; 11-11.5 min, 100 to 10% B; 11.5-14 min, 10% B.

Mass spectrometry conditions. Analysis was performed on a 5500 QTRAP mass spectrometer (SCIEX) used in positive/negative ion mode. Source temp: 450°C; Gas1/Gas2: 45; CUR: 40; ISVF: $\pm$ 4,500 V. Multiple reaction monitoring was used for detection.

MtDNA copy number detection. Cells were seeded in a 6-well plate at a density of approximately 3×10^5 cells per well. For KMO knockdown groups, after 24 h of culture, the cells were transfected with siKMO small interfering RNA or siControl RNA using Lipo8000® transfection reagent (Beyotime Biotechnology). For the siKMO + 3-HAA treated groups, upon reaching 70-80% confluence, the medium was changed to 2 ml culture medium containing 100 μ M 3-HAA or an equivalent volume of DMSO (control). The cells were then transfected with siKMO small interfering RNA. At 48 h after transfection, cell DNA was extracted using a DNA extraction kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. For KMO overexpression groups, DNA was extracted after 48 h of culture. mtDNA copy number was detected by qPCR using primers specific for the mitochondrial gene ND1 and HGB was used as reference gene.

Cellular ATP level measurement. Cells were lysed using ATP detection lysate, then centrifuged at 12,000 x g, for 5 min at 4°C. The supernatant was collected as the test sample. An ATP standard curve was generated by diluting the ATP standard solution (10 μ M) to concentrations ranging from 0.01-10 μ M. The ATP detection working solution was prepared at a 1:9 ratio of reagent to diluent. Working solution (100 μ l) was added to 96-well black plate and incubated at room temperature for 5 min. Subsequently, 20 μ l of sample or standard was added to each well, mixed and the relative light unit value was measured after 2 sec. ATP concentration was calculated using the standard curve.

Intercellular reactive oxygen species (ROS) level detection. Cells were collected in a 5 ml centrifuge tube. The ROS fluorescent probe (Bestbio Technology Co., Ltd.), 2,7-dichlorofluorescein acetate DCFH-DA, was prepared at a final concentration of 10 μ M (diluted 1:1,000 in serum-free DMEM). 500 μ l of the DCFH-DA working solution was added to each tube and the mixture was incubated at 37°C for 20 min in the dark. The unbound DCFH-DA probe was removed by washing with serum-free medium. Cells were re-suspended in DMEM and then analyzed by flow cytometry (FACSCelesta, BD Biosciences) with excitation at 488 nm (25). The results were analyzed using FlowJo 7.6 software (BD Biosciences).

Statistical analysis. Statistical analyses were performed using R (version 4.2.2, <http://www.R-project.org/>), GraphPad Prism (version 8.0; Dotmatics) and SPSS (version 25; IBM Corp.). All experiments were performed three times independently. The data were presented as the mean $\pm$ SD. The unpaired Student's t-test was employed for comparisons between two groups with normally distributed continuous variables, while Mann-Whitney U test was used for non-normally distributed data. One-way ANOVA with Tukey's post hoc test was used for comparisons among three groups. Correlations between continuous variables were assessed using Spearman's rank correlation analysis. Categorical variables were compared

between groups using the χ^2 test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Downregulation of KMO expression is related to the poor prognosis in HCC. Analysis of GSE101728, GSE45050, GSE84598 and GSE121248 datasets identified 31 upregulated and 109 downregulated genes commonly associated with HCC (Fig. S1A-C). Intersection with mitochondrial-related genes yielded one upregulated and 14 downregulated MRDEGs (Fig. S1D). Notably, KMO expression was consistently downregulated across all four datasets (LogFC ranging from -2.094 to -3.453; Fig. S1E). The finding was validated in TCGA data which demonstrated significantly lower KMO expression in HCC patients compared to normal individuals (Fig. 1A). qPCR and immunohistochemical results both showed that compared with matched adjacent normal tissues, the expression of KMO in HCC tumor tissues decreased (Fig. 1B and C). The present study grouped HCC tumor samples from TCGA database according to KMO median expression value, including 183 samples in KMO high expression group and 182 samples in KMO low expression group. Association analysis between KMO and clinicopathological features of HCC patients in TCGA database are shown in Table II. The results showed that KMO expression was related to Grade, T stage and tumor stage. Kaplan-Meier survival analysis demonstrated that the overall survival time of HCC patients with low KMO expression was significantly shorter than those with high expression (Fig. 1D).

The present study also analyzed the KMO expression within the HCC tumor microenvironment using single cell sequencing data. Analysis of 85,168 cells (28,998 genes) identified 10 cell types. Compared with normal tissues, HCC samples exhibited increased proportions of T cells, fibroblasts, epithelial and tumor cells, with decreases in endothelial cells, dendritic cell (DC), myeloid suppressor cell (MDSC) and natural killer cells (Fig. 1E). The expression of KMO in HCC was lower in HCC compared with that in normal (Fig. 1F) and was specifically reduced in DC, epithelial and tumor cells and MDSC (Fig. 1G), suggesting KMO may play important roles in these cells.

Downregulation of KMO in HCC cells promotes malignant progression of tumor. Using KMO knockdown and overexpression HCC cell models (Fig. S2), the present study evaluated the role of KMO in tumor progression. The CCK-8 test, EdU assay and colony formation experiment revealed that, compared with siControl group, the cell viability, cell proliferation and cell clone formation in siKMO group were increased significantly. Conversely, compared with empty vector (EV) control group, the cell viability, cell proliferation and cell clone formation in KMO-OE group were significantly decreased (Fig. 2). The scratch and Transwell assays demonstrated that cell migration ability in siKMO group were increased significantly, whereas the opposite effect was observed in KMO-OE group (Fig. 3). Flow cytometry analysis of cell apoptosis indicated that the apoptosis rate was increased in KMO-OE group compared with that in EV control group (Fig. S3).

Collectively, these results suggested that downregulation of KMO in HCC cells is associated with enhanced malignant progression.

KMO may localize to mitochondria and is associated with Try-kyn metabolism. WGCNA showed METurquoise module was correlated with KMO expression with a biggest correlation coefficient (0.45) and designating it as a key gene module that may be co-expressed with KMO (Fig. 4A). A total of 1,433 genes in METurquoise module were extracted for gene function enrichment analysis. GO enrichment analysis showed in biological processing, KMO co-expressed related genes participated in metabolic related pathways; in terms of cell components, KMO co-expression related genes are closely related to mitochondria; In terms of molecular function, KMO co-expression related genes may affect oxidoreductase activity, cofactor and coenzyme binding (Fig. 4B). These findings suggest KMO's potential role in many metabolic processes and its close association with mitochondrial structure and function, which was further supported by immunofluorescence experiments confirming the co-localization of KMO with mitochondria in HCC cells (Fig. 4C). KEGG enrichment results indicated that KMO co-expression related genes were related to Try metabolism (Fig. 4D). A PPI network constructed by six key genes of Try metabolic pathway from GSEA official website showed that KMO had direct interaction with IDO1, TDO2, IDO2, arylformamidase (AFMID), TDO2 and kynureninase (KYNU) with the correlation coefficients were 0, 0.47, 0.15, 0.37 and 0.47 respectively (Fig. 4E). These results suggest that KMO may reside in mitochondria and functionally interacts with other genes to influence the Try metabolic pathway.

KMO downregulation increases mitochondrial mass. Mitochondrial dysfunction is closely related to mitochondrial metabolic disorder and the occurrence and progression of HCC. Mito-Tracker staining was used to detect mitochondrial biogenesis and the 3D image statistical results showed that in MHCC-97H cells and LM3 cells, the mitochondrial mass in siKMO group was increased compared with that in siControl group. Conversely, KMO overexpression reduced mitochondrial mass relative to the EV control group (Fig. 5A and B). The present study detected the copy number of mtDNA coding gene ND1 to further verify whether KMO is related to mitochondrial biogenesis. The results showed that compared with siControl group, the ND1 copy number in siKMO group was increased significantly. Compared with EV group, ND1 copy number in KMO-OE group was decreased (Fig. 5C).

In addition, analysis of transcriptomic data revealed that all 13 genes encoded by the mitochondrial genome were upregulated in high-KMO expression patient samples and were positively correlated with KMO expression (Fig. 5D). Then the expression of two genes with the strongest correlation with KMO, MT-ATP6 and MT-CO1 was validated by using qPCR in KMO knockdown and overexpression HCC cells (Fig. 5E).

These results indicated that the downregulation of KMO in HCC cells promoted mitochondrial biogenesis. The uncoordinated changes in mitochondrial DNA copy number and mitochondrial gene expression suggested that KMO dysregulation can lead to imbalances in mitochondrial mass and function.

KMO downregulation promotes HCC cells proliferation and mitochondrial alterations by reducing 3-HAA production.

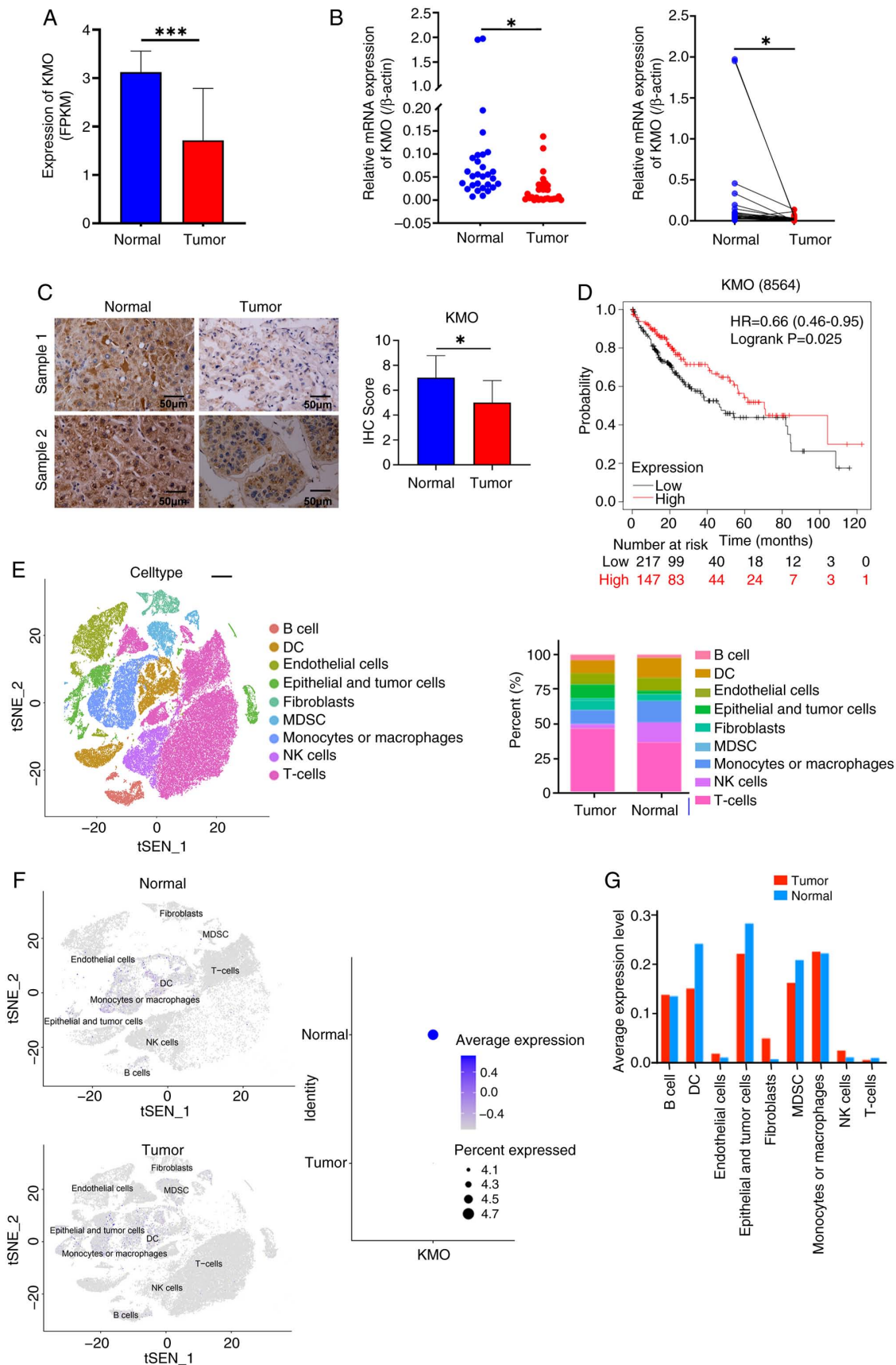


Figure 1. Downregulation of KMO expression is related to the poor prognosis in HCC. (A) mRNA expression of KMO in HCC tissue in TCGA database. (B) mRNA expression of KMO in cancer tissues and adjacent tissues of 31 HCC patients by using reverse transcription-quantitative PCR. (C) Immunohistochemistry showed the protein expression of KMO in HCC tissue and para-cancerous tissue. Scale bar, 50 μ m. Immunohistochemistry score was compared between two groups. (D) Survival analysis of KMO in HCC patients in TCGA database. (E) tSNE plot with annotations for cell types of HCC single-cell sequencing data analysis and the proportion of different cell types. (F) tSNE plot with KMO expression in tumor and normal samples. Bubble chart of KMO expression in two groups. (G) KMO expression in different cell types in HCC tumor and normal samples. * $P < 0.05$, *** $P < 0.001$. KMO, kynurenine 3-monooxygenase; HCC, hepatocellular carcinoma; TCGA, the Cancer Genome Atlas.

Table II. Relationship between KMO expression level and clinicopathological characteristics of patients with HCC in TCGA.

Variable	KMO Expression		P-value
	Low (n=182)	High (n=183)	
Age			0.716
≤60	88	85	
>60	94	98	
Sex			0.765
Male	124	122	
Female	58	61	
Child Pugh classification grade			0.252
Grade A	100	116	
Grade B and C	13	9	
N/A	69	58	
Grade			<0.001
Grade1-2	97	133	
Grade3-4	82	48	
N/A	3	2	
M			0.937
M0	138	125	
M1	1	2	
MX	43	56	
N			1.000
N0	124	125	
N1	2	2	
NX	56	56	
T			0.004
T1-2	123	148	
T3-4	57	34	
TX	2	1	
Stage			0.004
Stage I-II	113	142	
Stage III-IV	54	33	
N/A	15	8	
Survival status			0.559
Alive	114	120	
Dead	68	63	

KMO, kynurenine 3-monooxygenase; HCC, hepatocellular carcinoma; TCGA, the Cancer Genome Atlas.

In order to investigate how KMO influences Try metabolism, HPLC-MS methods were used to test the content of Try metabolites in HCC LM3 cells overexpressing KMO. Fig. S4 showed the total ion flow mass spectrometry of HCC cells. Among the total 16 metabolites detected, 3-HAA was the only one significantly elevated in HCC cells overexpressing KMO (Figs. S5 and 6A and B). Next, the expression of kynureninase (KYNU), the enzyme immediately downstream of KMO that

catalyzes the conversion of 3-hydroxykynurenine (3-HK) to 3-HAA was detected. No statistical significance was found in siKMO or KMO-OE group compared with their control groups (Fig. 6C), suggesting the changes in 3-HAA levels was directly mediated by KMO and independent of KYNU.

The present study further explored whether KMO downregulation induced cell proliferation is caused by 3-HAA. In siKMO HCC cells, 3-HAA can reverse the increased cell proliferation and vitality caused by KMO knockdown (Fig. 6D and E).

Similarly, it was investigated whether the mitochondrial changes induced by KMO are mediated by 3-HAA. As shown in Fig. 7A and B, in both MHCC97H and LM3 cells, after treatment with 3-HAA in siKMO cells for 48 h, the mitochondrial mass was decreased compared with the control group. The mtDNA copy number was significantly reduced in siKMO cells treated with 3-HAA compared to those treated with DMSO (Fig. 7C). ATP content in siKMO HCC cells was lower than that in control cells, while in KMO-OE HCC cells was higher. And 3-HAA can up regulate the ATP content decreased by KMO knockdown (Fig. 7D). ROS generation showed an opposite trend to ATP production and 3-HAA inhibited the ROS generation upregulated by KMO knockdown (Fig. 7E).

These results indicate that downregulation of KMO in HCC reduces the production of its downstream metabolite 3-HAA, thereby promoting HCC progression and inducing alterations in mitochondrial mass and function.

KMO downregulation reduces the expression of nuclear receptor subfamily 4 group A member 1 (NR4A1) and promotes its mitochondria translocation in a 3-HAA-dependent manner. To investigate the mechanisms underlying the mitochondrial alterations induced by KMO downregulation, the present study screened mitochondrial transcription factors associated with KMO expression. A total of 1,670 transcription factor genes downloaded from GTRD website (<http://gtrd.biouml.org>), were intersected with 2,030 mitochondrial-related genes and 1,721 DEGs between KMO high and low groups (26). A total of four KMO related mitochondrial transcription factors were obtained (Fig. 8A). In these four transcription factors, the expression of KMO was positively correlated with the expression of FOXO1 and NR4A1, while negatively correlated with the expression of HMGA1 and SOX4 (Fig. 8B). After qPCR verification, only NR4A1 expression was consistent with the data analysis: It positively correlated with KMO expression in HCC cells and 3-HAA treatment rescued in the downregulation of NR4A1 expression caused by KMO knockdown (Fig. 8C). Low NR4A1 expression was associated with poor prognosis in HCC patients (Fig. 8D). Similar to KMO, NR4A1 expression was positively correlated with the expression of ten mitochondrial genome-encoded genes (Fig. 8E).

Since NR4A1 is a mitochondria-associated transcription factor, the present study was interested in finding out the subcellular localization of NR4A1. After immunofluorescence staining, it was observed that KMO expression in HCC cells was distributed in both nucleus and cytoplasm. Combined with fluorescent staining of mitochondrial Mito-Tracker, NR4A1 showed increased expression in mitochondria in KMO knockdown HCC cells, whereas NR4A1 expression in mitochondria was reduced after overexpression of KMO. 3-HAA reversed

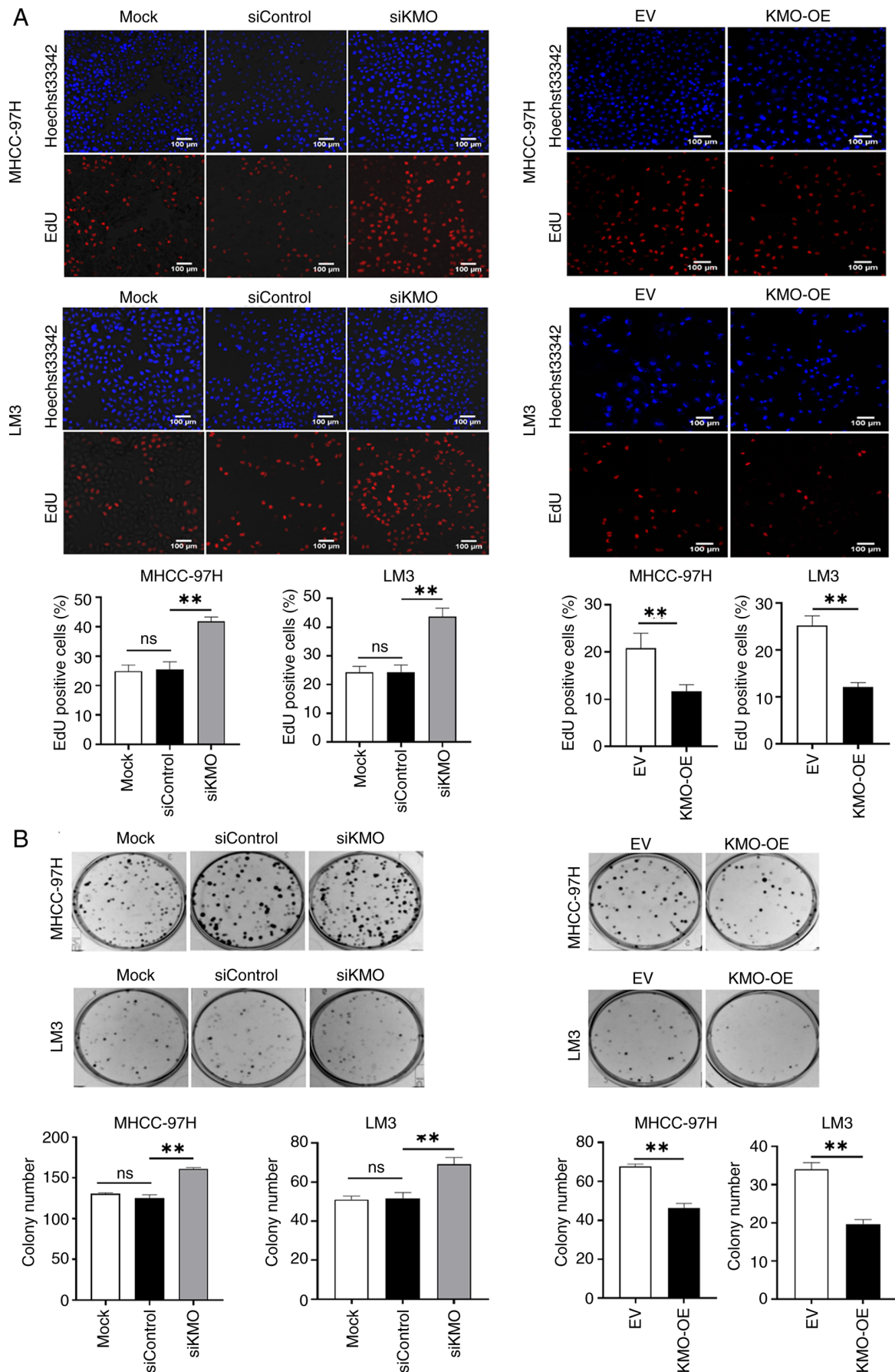


Figure 2. Downregulation of KMO promotes HCC cell growth. (A) Effect of KMO on proliferation activity of HCC cells by using EdU detection. EdU labeled newly proliferated cells (red) and Hoechst33342 labeled cell nucleus (blue). Positive cells rate (red/blue) were counted and compared between control and KMO-knockdown or overexpression groups. Scale bar, 100 μ m. (B) Effect of KMO on colony-forming ability of HCC cells. Colony number was counted and compared between control and KMO-knockdown or overexpression groups. ** $P < 0.01$. KMO, kynurenine 3-monooxygenase; HCC, hepatocellular carcinoma.

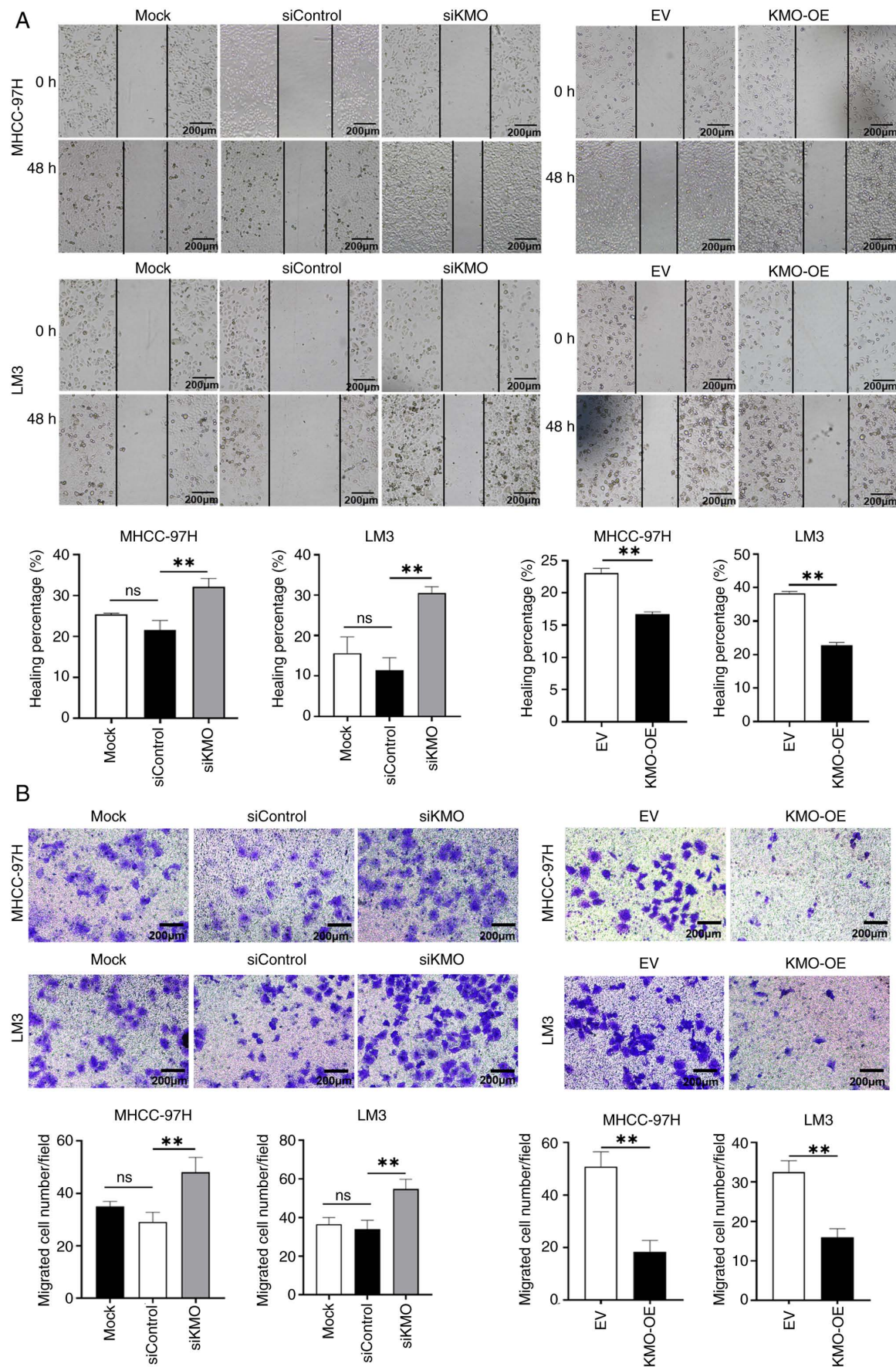


Figure 3. Downregulation of KMO promotes HCC cell migration. (A) Effect of KMO on migration of HCC cells by using wound healing assay. Scale bar, 200 μ m. Healing percentage was calculated and compared between control and KMO-knockdown or overexpression groups. (B) Effect of KMO on HCC cell migration by using Transwell chamber assay. Scale bar, 200 μ m. Migrated cell number was counted and compared between control and KMO-knockdown or overexpression groups. ** $P < 0.01$. KMO, kynurenine 3-monooxygenase; HCC, hepatocellular carcinoma; si, short interfering; EV, control group; OE, overexpressing.

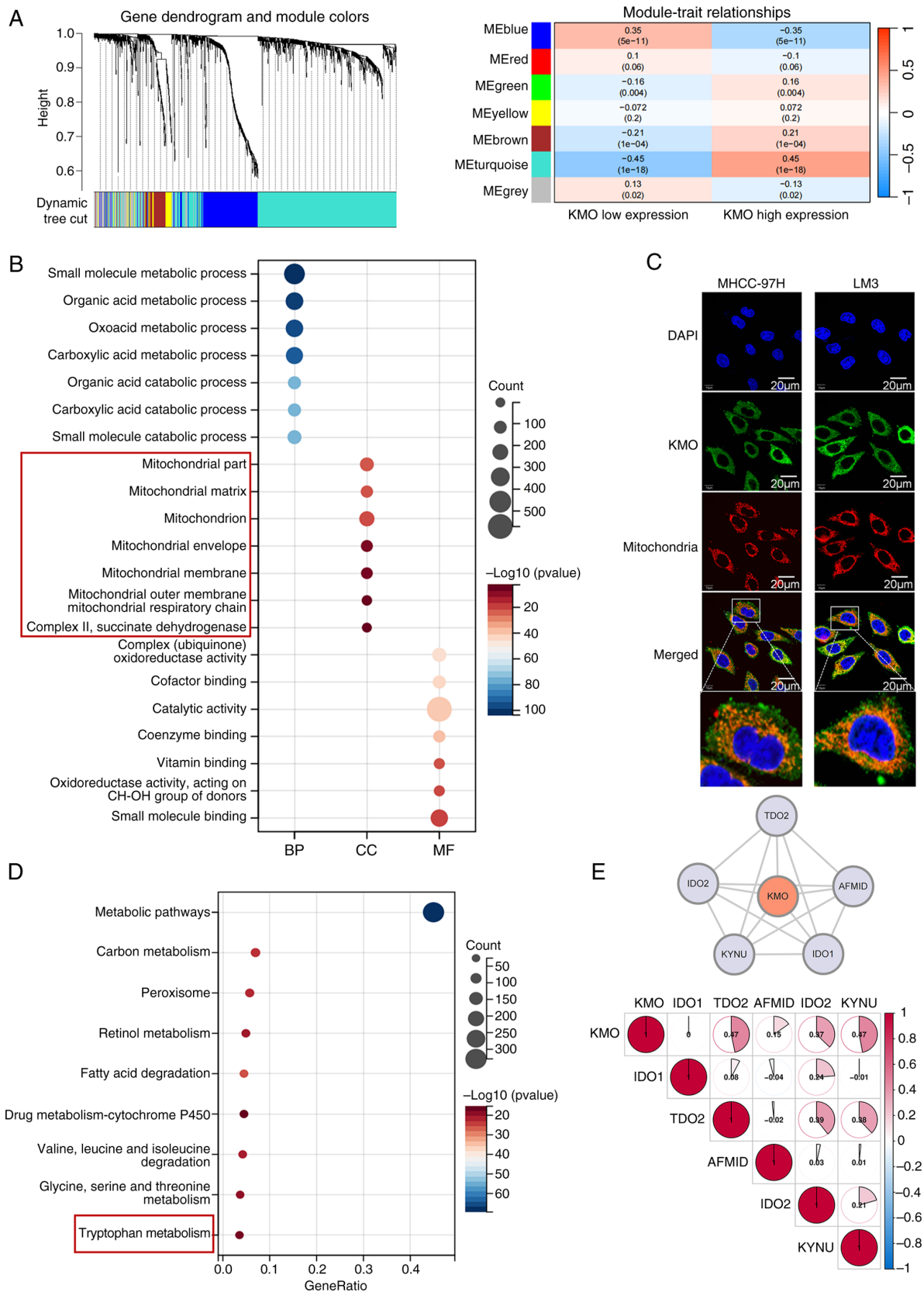


Figure 4. KMO locates in mitochondria and is related to Try-Kyn metabolism. (A) Weighted gene co-expression network analysis of KMO-related genes. Dendrogram shows the size of different color modules. The numerical value represents the correlation coefficient and the values in parentheses represent the significance P-value. Genes in MEturquoise module have the strongest correlation with KMO expression (correlation coefficient=0.45). (B) GO enrichment analysis of KMO-related co-expressed genes. The GO cellular component analysis showed that these genes were enriched in different mitochondrial components (Red box). (C) Subcellular distribution of KMO in HCC cells observed by using confocal microscopy after immunofluorescence staining. KMO is shown in green and mitochondria are stained red. The yellow color indicates the co-localization of KMO with mitochondria. Scale bar, 20 μ m. (D) KEGG enrichment analysis of KMO-related co-expressed genes. These genes can be enriched in Try metabolism signaling pathway (Red box). (E) The protein-protein interaction network and correlation between KMO and key genes involved in Try metabolism pathway. KMO, kynurenine 3-monooxygenase; Try, tryptophan; Kyn, kynurenine; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

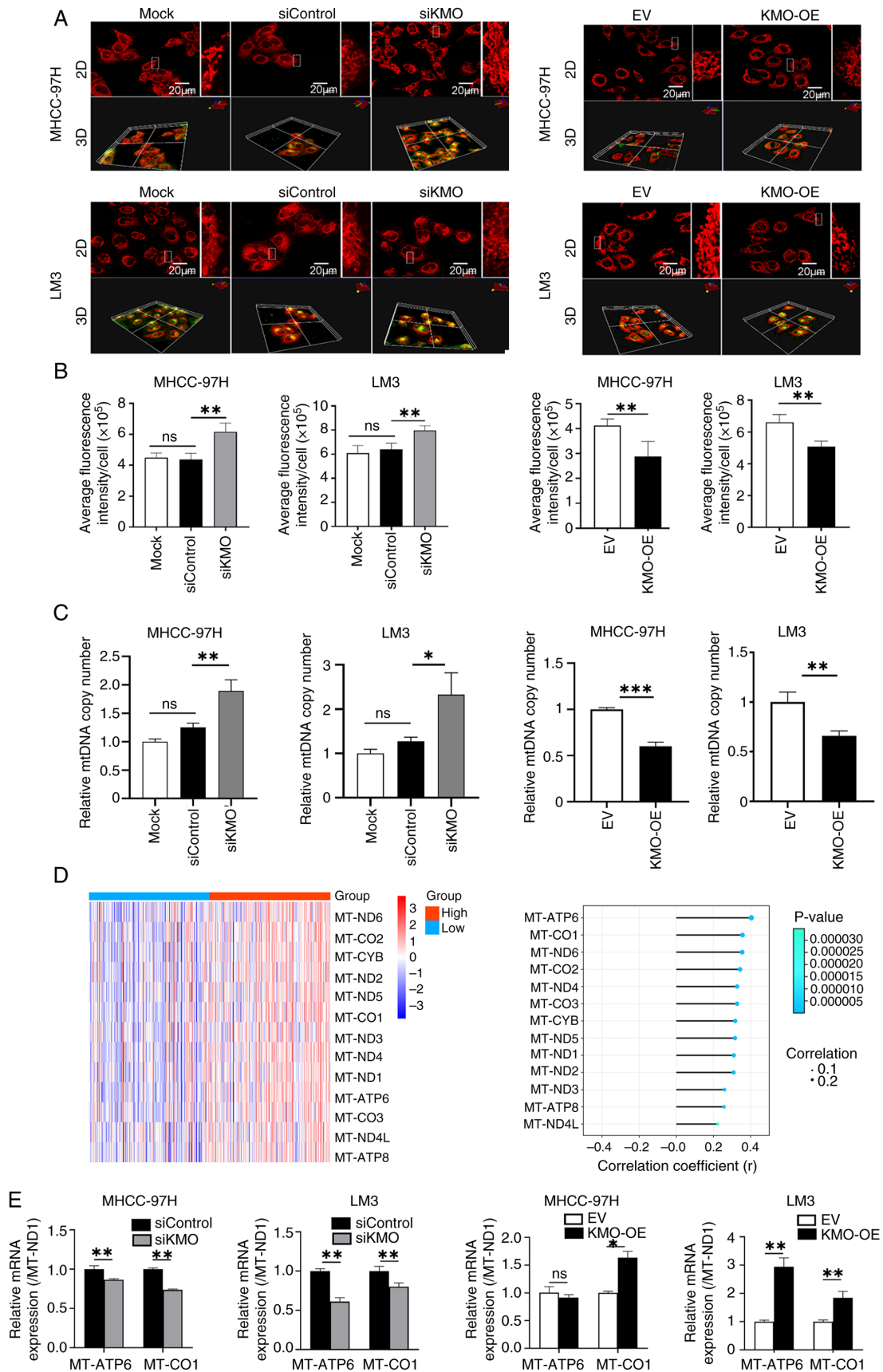


Figure 5. Downregulation of KMO causes disorder of mitochondrial mass and function. (A) Effect of KMO on mitochondrial mass of HCC cells was observed by using confocal microscopy after MitoTracker staining. 2D and corresponding 3D images were collected and the parts in white box in the 2D image were magnified. Scale bar, 20 μ m. (B) Mitochondrial mass was analyzed by using the value of average fluorescence intensity per cell. (C) Effect of KMO on mtDNA copy number in HCC cells was assayed by using reverse transcription-quantitative PCR for MT-ND1. (D) Heatmap showed the expression of 13 genes encoded by the mitochondrial genome in the high and low KMO expression groups. Lollipop chart showed the correlation between KMO and these 13 genes. (E) Reverse transcription-quantitative PCR verification of mRNA expression levels of MT-ATP6 and MT-CO1 genes in HCC cells with KMO knockdown and overexpression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. KMO, kynurenine 3-monooxygenase; si, short interfering; EV, control group; OE, overexpressing.

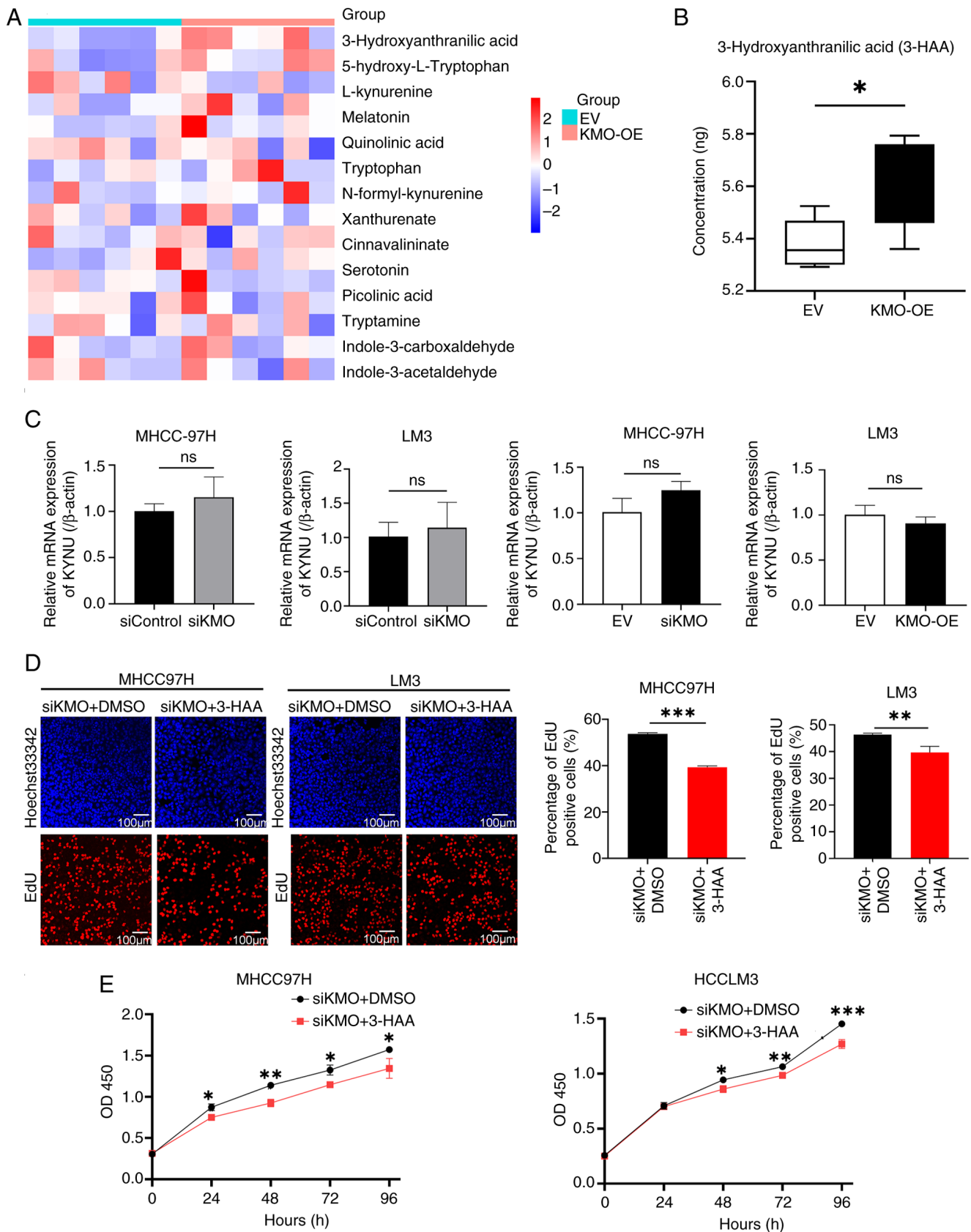


Figure 6. Downregulation of KMO induced the decrease of 3-HAA level promotes HCC cells proliferation. (A) Heatmap showed tryptophan metabolites levels in KMO overexpression and control HCC cell groups. (B) Comparison of 3-HAA levels between KMO overexpression group and control group. (C) mRNA expression of KYNU in KMO knockdown and overexpression HCC cells by using reverse transcription-quantitative PCR. (D) Effect of 3-HAA on cell proliferation in KMO knockdown HCC cells by using EdU detection. EdU labeled newly proliferated cells (red) and Hoechst33342 labeled cell nucleus (blue). Positive cells rate (red/blue) were counted and compared between KMO-knockdown cells treated with DMSO (control) and KMO-knockdown cells treated with 3-HAA groups. Scale bar, 100 μ m. (E) Effect of 3-HAA on cell viability in KMO knockdown HCC cells by using CCK8 test on 0, 24, 48, 72 and 96 h. The cell proliferation rates of KMO knockdown cells treated with 3-HAA or DMSO at the same time were compared. Final working concentration of 3-HAA: 100 μ M. * P <0.05, ** P <0.01, *** P <0.001, ns, P >0.05. KMO, kynurenine 3-monooxygenase; 3-HAA, 3-hydroxyanthranilic acid; KYNU, kynureninase.

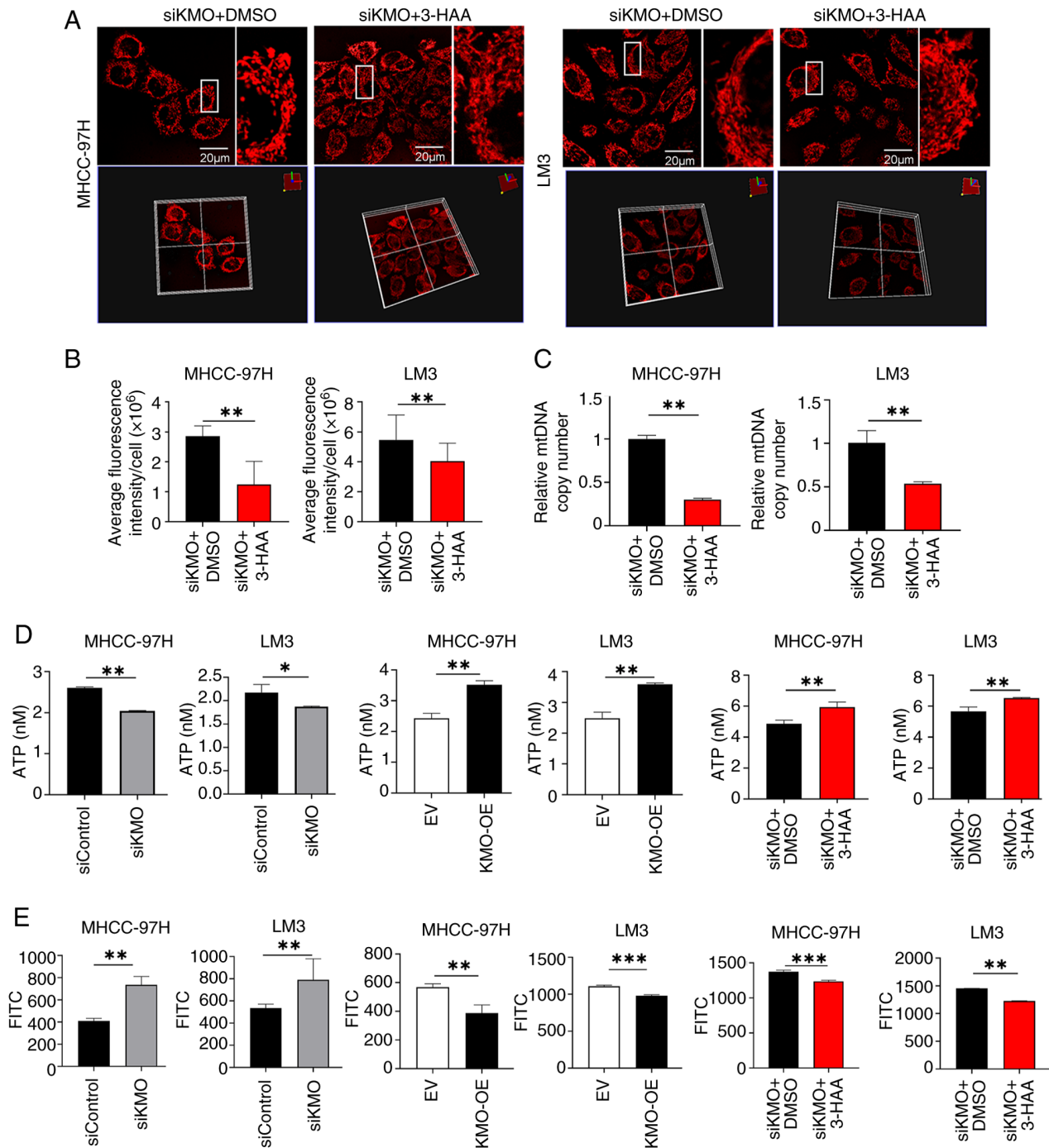


Figure 7. Downregulation of KMO promotes disorder of mitochondrial mass and function via 3-HAA in HCC cells. (A) Effect of 3-HAA on mitochondrial mass in KMO knockdown HCC cells was observed by using confocal microscopy after MitoTracker staining. The parts in white box were magnified. Scale bar, 20 μ m. (B) Mitochondrial mass was analyzed by using the value of average fluorescence intensity per cell. (C) Effect of 3-HAA on mtDNA copy number in KMO knockdown HCC cells. (D) Effect of 3-HAA on ATP production in KMO knockdown HCC cells. (E) Effect of 3-HAA on ROS production in KMO knockdown HCC cells. (Final working concentration of 3-HAA: 100 μ M. * P <0.05, ** P <0.01, *** P <0.001. KMO, kynurenine 3-monooxygenase; 3-HAA, 3-hydroxyanthranilic acid; HCC, hepatocellular carcinoma.

the mitochondrial translocation of NR4A1 induced by KMO knockdown (Fig. 9).

These results illustrated that downregulation of KMO in HCC cells can lead to reduced NR4A1 expression and promotes its mitochondrial translocation, both of which are mediated by 3-HAA.

The decrease of NR4A1 induced by downregulation of KMO promotes mitochondrial biogenesis and malignant growth

of HCC cells. In KMO overexpressed HCC cells, the expression of NR4A1 was knocked down to investigate the effect of KMO-mediated NR4A1 on HCC cell growth and mitochondrial mass and function. CCK8 and EdU results showed that silencing of NR4A1 enhanced cell viability and proliferation in HCC cells overexpressing KMO (Fig. 10A and B). NR4A1 knockdown increased mitochondrial mass in HCC cells overexpressing KMO compared with siControl cells (Fig. 10C). Additionally, ATP production also decreased by NR4A1

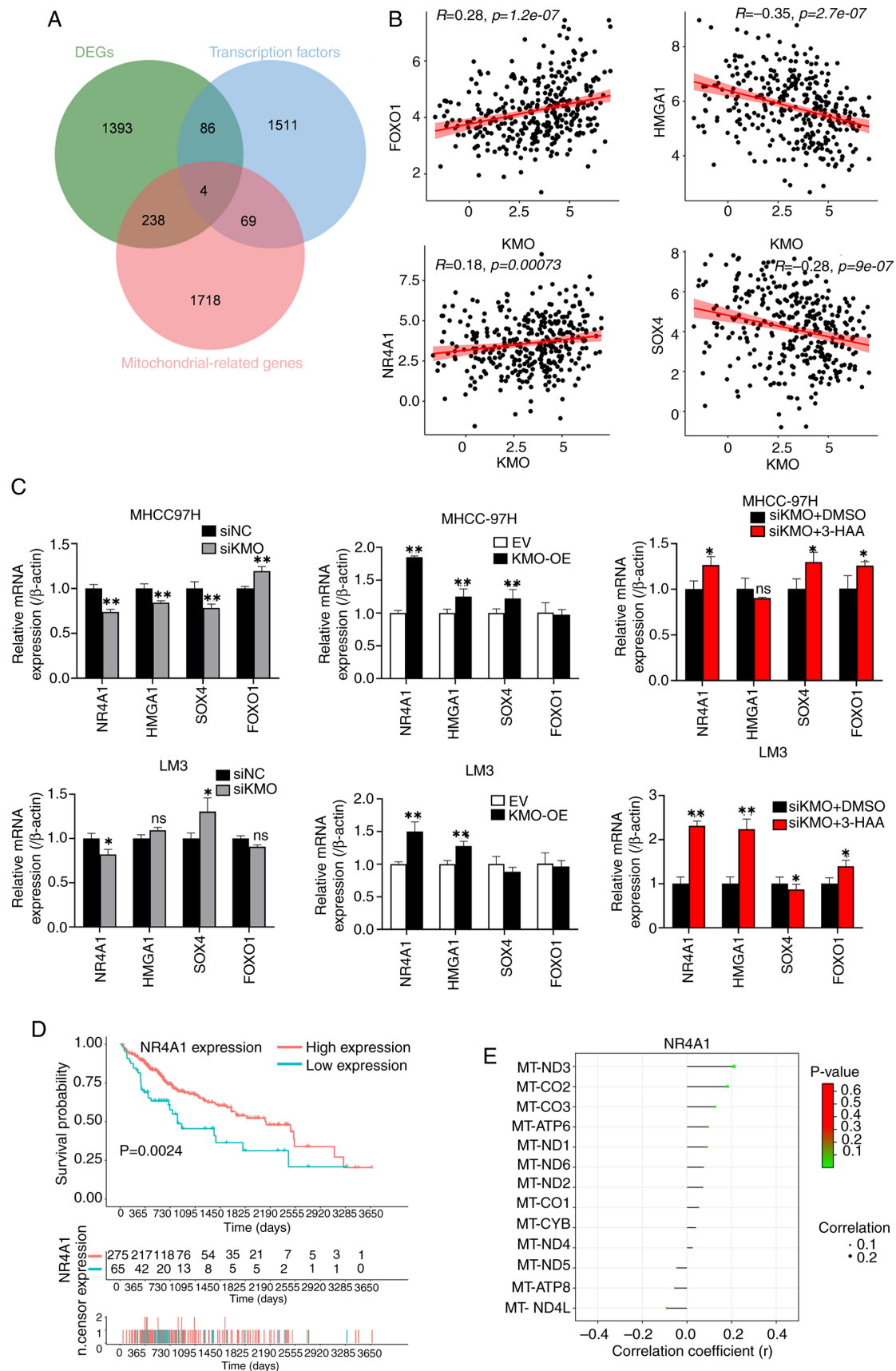


Figure 8. Downregulation of KMO promotes the decrease of NR4A1 in HCC cells. (A) Venn diagram among KMO related DEGs, transcription factors and mitochondrial related genes. The overlapped four genes are the screened mitochondrial related transcription factors from KMO related DEGs. (B) Correlation analysis between KMO and four mitochondrial related transcription factors screened using TCGA database. (C) mRNA expression of four mitochondrial related transcription factors in KMO-knockdown, overexpression and 3-HAA treated KMO-knockdown HCC cells and the corresponding controls. (D) Survival analysis of NR4A1 in HCC patients in TCGA database. (E) The correlation analysis between NR4A1 and mitochondrial DNA-encoded genes in HCC. Final working concentration of 3-HAA: 100 μ M. * $P < 0.05$, ** $P < 0.01$, ns: $P > 0.05$. KMO, kynurenine 3-monooxygenase; NR4A1, nuclear receptor subfamily 4 group A member 1; HCC, hepatocellular carcinoma; DEGs, differentially expressed genes; TCGA, the Cancer Genome Atlas; 3-HAA, 3-hydroxyanthranilic acid.

knockdown in KMO overexpressed HCC cells (Fig. 10D). These results indicated that NR4A1 inhibition promotes mitochondrial biogenesis and the malignant growth of HCC cells.

Discussion

Abnormalities in Try metabolism, as well as mitochondrial structure and function, are involved in the development of HCC. As a key enzyme in the Try metabolic pathway, KMO co-localizes with mitochondria. However, to the best of the authors' knowledge, the effects of KMO on mitochondrial mass and function in HCC have not yet been reported. The present study identified that KMO expression was downregulated in HCC patients through multiple public datasets and further validated this finding in tissue samples. Furthermore, KMO downregulation in HCC cells was shown to promote tumor cell proliferation and migration. The co-localization of KMO and mitochondria was determined in HCC cells and the downregulation of KMO promoted mitochondrial biogenesis. Mechanistically, low expression of KMO in HCC cells reduced 3-HAA levels, which suppressed expression of the transcription factor NR4A1 and drove its mitochondrial translocation, thereby disrupting mitochondrial homeostasis and promoting the proliferation of HCC cells (Fig. 10E).

Try metabolism through the Kyn pathway serves an immunosuppressive role in liver cancer (27). However, the distinction between the immunomodulatory effects of Try itself and the effects of downstream products of Try metabolism remains unclear (28). KMO is involved in Try metabolism through the Kyn pathway, which exists mainly in the liver and ~95% of Try is oxidatively degraded via this pathway (29). In the current study, multiple datasets and experiments validated that KMO expression was reduced in HCC and was associated with a poor prognosis in patients with HCC. In HCC cells with KMO knockdown and overexpression, it was also verified that reduction of KMO promoted the malignant progression of HCC tumor cells. These results are consistent with the findings of Shi *et al* (20). A possible theory is that the downregulation of KMO disrupts the Kyn pathway and induces the accumulation of Kyn, which suppresses immune cell populations and increases tumor growth (30). Notably, it may also promote tumor progression by affecting the generation of downstream products of the Kyn pathway. In addition, the results of single-cell sequencing data analysis revealed that KMO expression was downregulated in DC, epithelial and tumor cells and MDSC from HCC tissue, suggesting that KMO expression in DC, epithelial and tumor cells and MDSC has an antitumor effect and requires further study.

KMO has previously been reported to be localized on the outer mitochondrial membrane of pig liver (9). A previous study found that inhibition of KMO can effectively inhibit cardiomyocyte apoptosis and ferroptosis by regulating mitochondrial fission and fusion (31). Chen *et al* (32) demonstrated that KMO, as a key gene for mitochondrial dysfunction, has important diagnostic value for intracranial aneurysms. Downregulation of KMO expression has also been found to enhance mitochondrial fusion and inhibit mitophagy in ischemic stroke (33). These studies have revealed that KMO can be involved in disease development by affecting mitochondrial structure or function. The present study confirmed

the co-localization of KMO with mitochondria in HCC cells. Furthermore, GO enrichment analyses of KMO co-expressed genes showed that these genes were related to mitochondrial structural components and in HCC cells, the expression of KMO affected the mitochondrial mass, mitochondrial biogenesis and expression of mitochondrial encoded genes. In particular, the opposability of mitochondrial mass to mitochondrial encoded gene expression points to a state of mitochondrial dysfunction.

Try is initially metabolized by IDO1, IDO2 and TDO to produce Kyn. One of the three Kyn metabolic pathways includes the catalysis of Kyn by KMO to form 3-HK, which is subsequently converted to 3-HAA by KYNU. 3-HAA is further metabolized by 3-hydroxyl amino acid oxygenase to generate quinolinic acid (QUIN). QUIN serves as a substrate for the production of nicotinamide adenine dinucleotide, which is involved in a variety of important physiological processes, such as intracellular energy metabolism, DNA repair and signal transduction (34). Notably, KMO expression has the potential to influence its downstream metabolites. Since KMO expression was downregulated in HCC, the current study chose HCC cells overexpressing KMO to detect Try metabolites. The results showed that only 3-HAA was increased in HCC cells overexpressing KMO compared with that in the control cells, whereas 3-HK, a direct metabolite of KMO, was not detected. The reason for this phenomenon may be that KYNU has a strong metabolizing ability for 3-HK, while both silencing and overexpression of KMO did not affect KYNU expression. It may be assumed that upregulated KMO mainly enhanced 3-HAA levels. It has previously been reported that the Kyn derivative 3-HAA is lower in HCC cells and exogenous 3-HAA induces the apoptosis of HCC cells by binding the transcription factor YY1 (24). In a mouse model of HCC, 3-HAA treatment has been reported to inhibit HCC growth by regulating the function of macrophages (35). Gan *et al* (36,37) also demonstrated that 3-HAA may sensitize HCC cells to sorafenib by reducing AKT phosphorylation, promoting apoptosis and suppressing tumor cell stemness. In conclusion, 3-HAA could inhibit HCC progression through multiple mechanisms, including the induction of apoptosis and reduction of stemness, indicating that it may serve as a promising therapeutic target for HCC.

The present study also observed that 3-HAA could reverse the malignant phenotype of HCC cells caused by KMO downregulation. In addition, 3-HAA-treated cancer cells exhibited a reversal of the changes in mitochondrial mass, mitochondrial biogenesis, ATP and ROS production caused by KMO. The possible mechanisms underlying how 3-HAA could ameliorate HCC were elaborated from a new perspective in the present study. It has previously been reported that M2 macrophage-secreted KYNU and 3-HAA may upregulate the expression of superoxide dismutase 2 to decrease mitochondrial ROS in endometrial cancer. Although this previous study concluded that high KYNU and 3-HAA promote endometrial cancer progression, it also provides valuable evidence for the association between 3-HAA and mitochondria (38). Ruan *et al* (39) reported that bronchopulmonary dysplasia (BPD) was associated with markedly reduced 3-HAA levels and exhibited typical ferroptosis-associated changes, such as mitochondrial pyknosis and loss of cristae, whereas 3-HAA

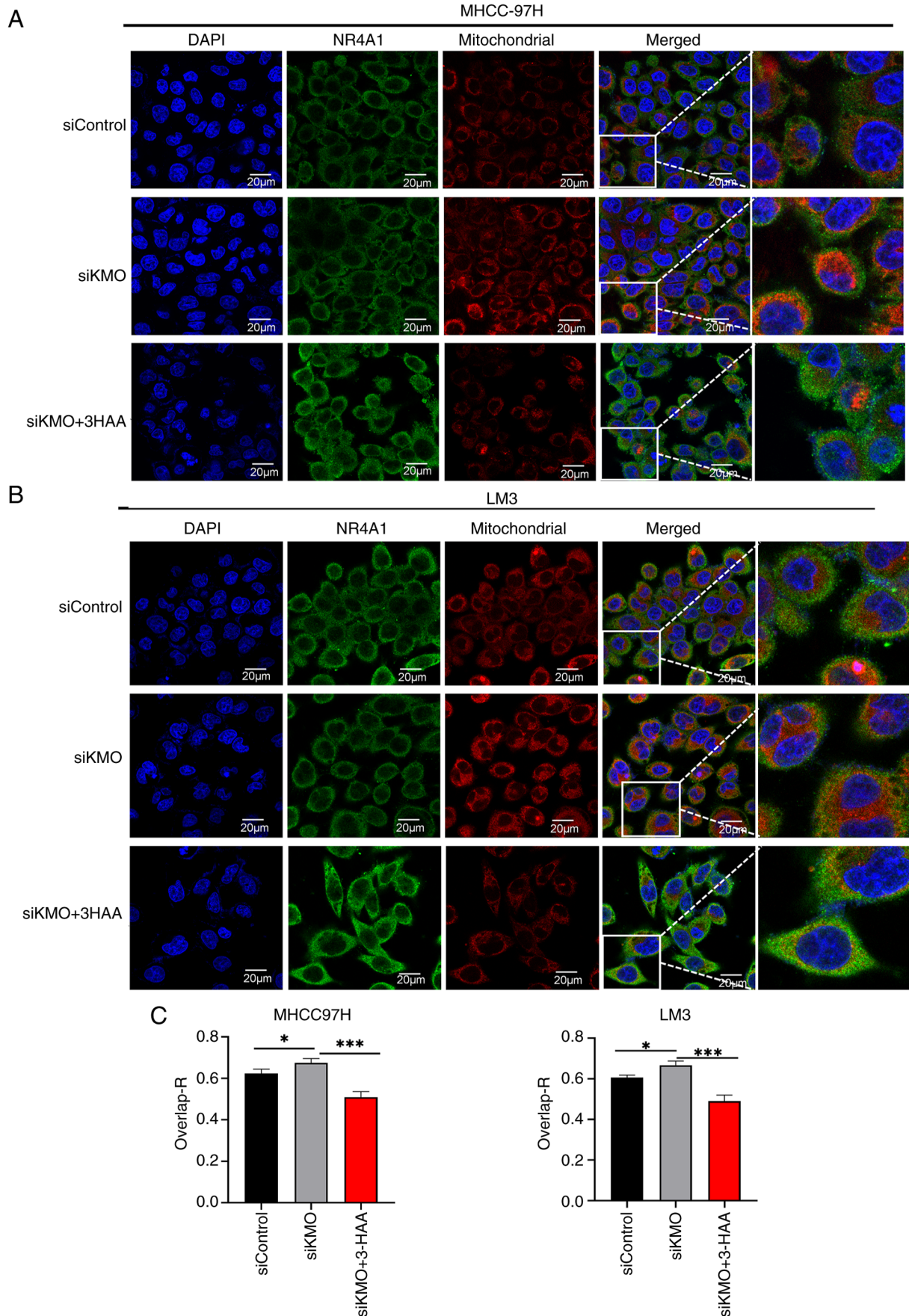


Figure 9. Downregulation of KMO promotes translocation of NR4A1 to mitochondria by 3-HAA. Effect of KMO and 3-HAA on subcellular distribution of NR4A1 in (A) MHCC-97H and (B) LM3 cells. NR4A1 is shown in green and mitochondria are stained red. The yellow color indicates the co-localization of NR4A1 with mitochondria. Scale bar, 20 μ m. (C) The overlap rate of NR4A1 and mitochondria (orange color rate) was quantified and compared between the indicated HCC cells. Final working concentration of 3-HAA: 100 μ M. Confocal microscope magnification, x100. * P <0.05, *** P <0.001. KMO, kynurenine 3-monooxygenase; NR4A1, nuclear receptor subfamily 4 group A member 1; 3-HAA, 3-hydroxyanthranilic acid; HCC, hepatocellular carcinoma.

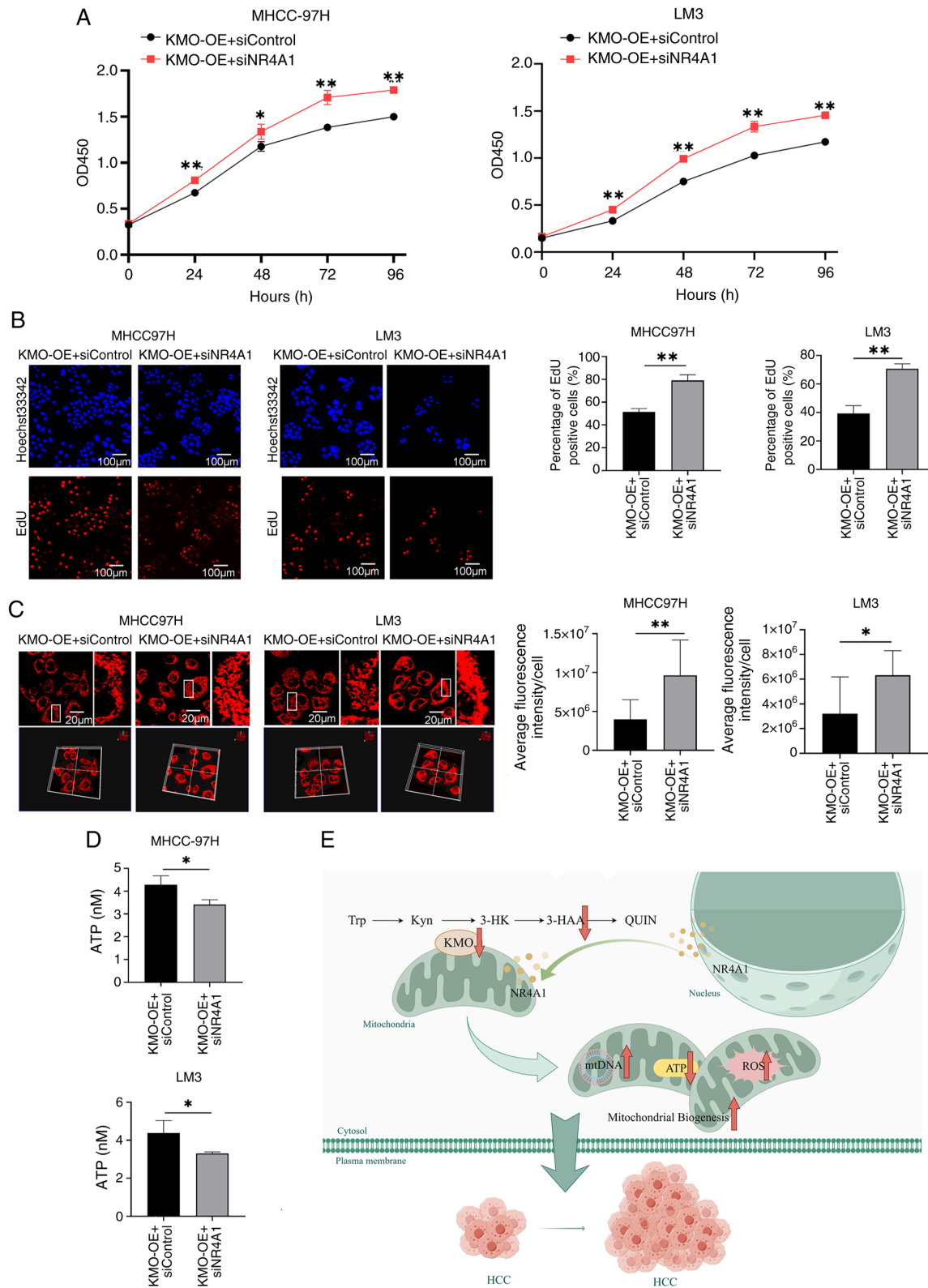


Figure 10. KMO affects cell growth, mitochondrial quality and ATP production through NR4A1. (A) Effect of NR4A1 downregulation on cell viability in KMO overexpression HCC cells was observed by using CCK8 test on 0, 24, 48, 72 and 96 h. The cell viability rates between KMO overexpression cells transfected with siKMO and transfected with siControl were compared. (B) Effect of NR4A1 downregulation on cell proliferation in KMO overexpression HCC cells was observed by using EdU test. EdU labeled newly proliferated cells (red) and Hoechst33342 labeled cell nucleus (blue). Scale bar, 100 μ m. Positive cells rate (red/blue) were counted and compared between KMO overexpression cells and KMO overexpression cells with NR4A1 knockdown. (C) Effect of NR4A1 downregulation on mitochondrial mass in KMO overexpression HCC cells was observed by using confocal microscopy after MitoTracker staining. The parts in white box were magnified. Scale bar, 20 μ m. Mitochondrial mass was analyzed by using the value of average fluorescence intensity per cell. (D) Effect of NR4A1 downregulation on ATP production in KMO overexpression HCC cells. (E) The mechanism of KMO downregulation promoting tumor growth in HCC: KMO downregulation reduced 3-HAA levels, which suppressed expression of the transcription factor NR4A1 and drove its mitochondrial translocation, thereby disrupting mitochondrial homeostasis and promoting the proliferation of HCC cells. * $P < 0.05$, ** $P < 0.01$. KMO, kynurenine 3-monooxygenase; NR4A1, nuclear receptor subfamily 4 group A member 1; HCC, hepatocellular carcinoma; 3-HAA, 3-hydroxyanthranilic acid; ROS, reactive oxygen species; 3-HK, 3-hydroxykynurenine; QUIN, quinolinic acid; Trp, tryptophan; Kyn, kynurenine.

treatment could attenuate these mitochondrial abnormalities in type II alveolar epithelial cells, suggesting 3-HAA as a potential target for treating BPD.

To further clarify the regulatory mechanism by which KMO downregulation mediates the reduction of 3-HAA affecting mitochondrial function, the present study explored the mitochondria-related transcription factors that may be affected by KMO expression. As a result, NR4A1 was revealed to be positively correlated with KMO expression in HCC cells. NR4A1, also known as Nur77, affects tumorigenesis and tumor progression via various cellular processes, such as proliferation, endoplasmic reticulum stress, apoptosis and autophagy (40,41). Based on the cancer type and stage, NR4A1 can exhibit both pro-tumorigenic and tumor-suppressive effects via transcription-dependent and transcription-independent manners (41). In liver cancer, the low expression of NR4A1 can promote HCC progression by increasing glycolysis, inhibiting apoptosis or regulating β -catenin expression (42-44). In the current study, downregulated NR4A1 was associated with poor survival in patients with HCC and NR4A1 was also shown to be positively related to the expression of the majority of mtDNA encoded genes, such as MT-ATP6 and MT-ND1, thus suggesting that low expression of NR4A1 in HCC also affects mitochondrial function. It has been reported that NR4A1 promotes apoptosis in HCC cells when its cytoplasmic expression is increased (44). NR4A1 translocated to the mitochondria can promote the transformation of the anti-apoptotic protein Bcl-2 into a pro-apoptotic state, interfere with mitochondrial division and fusion, inhibit mitochondrial autophagy and lead to irreversible mitochondrial damage and cell apoptosis (45). In a model of myocardial ischemia-reperfusion injury, NR4A1 has been shown to be elevated and may promote mitochondrial fission by regulating mitochondrial fission 1 protein (46). Furthermore, the synergistic effect of NR4A1 and YY1 on macrophages can increase the abundance and activity of mitochondria (47). However, in Parkinson's disease research, it has been reported that NR4A1, which shuttles from the nucleus to the mitochondria, can inhibit apoptosis by stabilizing the presenilin-associated mitochondrial like protein (PARL)-Bcl-2 complex, thereby overexpression of NR4A1 can restore PARL-mediated anti-apoptotic signaling for therapeutic purposes (48). In the present study, the subcellular localization of NR4A1 was assessed in siKMO HCC cells to determine the effect of KMO on NR4A1 translocation. Unexpectedly, compared with in the control cells, although the expression of NR4A1 was reduced, co-localization with mitochondria was increased in siKMO HCC cells. The reasons for this phenomenon are likely to be complex: Total NR4A1 expression was reduced after KMO interference; however, mitochondrial mass was increased, which possibly enhanced shuttling of NR4A1 from the nucleus to the mitochondria. When siKMO HCC cells were treated with 3-HAA, the expression of NR4A1 was increased and co-localization with the mitochondria was decreased, implying that KMO may regulate the expression and translocation of NR4A1 induced by 3-HAA. NR4A1 exerts an inhibitory effect on cell proliferation in the nucleus and its reduction and mitochondrial translocation will promote HCC cell proliferation; however, whether NR4A1 promotes apoptosis or inhibits apoptosis in the mitochondria requires an improved experimental design for verification. It may play

a role in promoting apoptosis; however, due to the reduction in NR4A1, although mitochondrial co-localization is enhanced, the overall effect on HCC cell proliferation may be more notable than the effect on apoptosis. In addition, the pro-apoptotic function of mitochondrial NR4A1 may be abrogated due to its critically low overall expression level. The subsequent cellular experiments in the present study also demonstrated that in HCC cells overexpressing KMO, downregulation of NR4A1 expression promoted tumor cell proliferation, increased mitochondrial mass and decreased ATP. These findings indicated the importance of KMO, 3-HAA and NR4A1 in liver carcinogenesis; however, further studies are needed to elucidate the underlying mechanism using a broader panel of HCC cell lines and rescue experiments.

By employing a multi-faceted approach, the rigor and validity of the current conclusions were strengthened. The cellular mechanistic studies complemented those of public large-scale human HCC transcriptomics datasets (TCGA and GEO datasets), enhancing the clinical relevance of the study. The present data showed that in patients with HCC and in HCC cell models, the lack of KMO may lead to the progression of HCC. Therefore, targeting KMO and related molecules, as well as the associated signaling pathways, may be a promising therapeutic strategy for HCC. The feasible strategies include but are not limited to: i) Based on the bulk mRNA sequencing data of the adult population, the drug sensitivity in the KMO low and high expression groups could be predicted and patients with HCC may choose more precision treatment drugs according to KMO expression; ii) KMO downstream metabolites, such as 3-HAA, as small molecule compounds, have not yet become approved therapeutic drugs, but 3-HAA is a potential candidate drug that is currently undergoing extensive research; iii) concurrent inhibition of the upstream enzyme IDO1/TDO and enhancement of the downstream enzyme KMO in Try metabolism could exert a synergistic effect, leading to a more potent reversal of immunosuppression through the reduction of Kyn buildup.

The present study focused on investigating the influence of the key enzyme in Try metabolism, KMO, on disordered mitochondria in the development of HCC. Despite yielding valuable insights, several limitations remain. Firstly, the study was conducted at the cellular level based on publicly available datasets and lacked experimental validation at the animal level. Second, the mechanism of NR4A1 translocation induced by KMO or 3-HAA requires further exploration. Third, further experiments need to be designed and performed to investigate the differential functions of NR4A1 in the nucleus and mitochondria of HCC cells and animal models. Fourth, the method for detecting mitochondrial morphology in this study was relatively limited, it is necessary to employ a variety of further detection methods to determine the changes of mitochondrial morphology and structure. Finally, it will be necessary to assess the mechanism underlying the effects of KMO downregulation on increased mitochondrial mass and dysfunction in the future.

In conclusion, the present study indicated that low KMO expression in HCC may affect mitochondrial mass and function by reducing the level of the Try metabolite 3-HAA and downregulating the expression of NR4A1 and promoting its mitochondrial translocation, which in turn could promote the

progression of HCC. KMO agonists are expected to achieve two goals simultaneously in alleviating HCC: Restoring the Try metabolic pathway and enhancing NR4A1 expression. The present findings provide new insights into the treatment of HCC, potentially targeting the mitochondria and the Try-Kyn pathway.

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Availability of data and materials

The GEO datasets are available for download from the National Center for Biotechnology Information online Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>; GSE101728, GSE45050, GSE84598 and GSE121248). TCGA-LIHC data was downloaded from The Cancer Genome Atlas (TCGA; (<https://portal.gdc.cancer.gov/>; TCGA-LIHC) and relative phenotype and survival data are from University of California Santa Cruz (UCSC) [<https://xenabrowser.net/datapages/>, TCGA Liver Cancer (LIHC)]. Cell annotation lists are available online at Cell Marker 2.0 (<http://117.50.127.228/CellMarker/>). R and other custom scripts for analyzing the data may be requested from the corresponding author.

Authors' contributions

ML, HW and JZ designed the experiments, acquired and interpreted the data, and drafted the manuscript. XZ and YH completed the data analysis and cell experiments. LN, LG and FL collected the HCC tissue samples, completed the tissue experiments, and interpreted the data. GZ, FJ and LL analyzed, checked the data, and edited the manuscript. ML and JZ confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of the First Hospital of Shanxi Medical University (approval number: 2021SLLO86). All patients understood and signed the informed consent. All patients consent for publication.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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