



The MIF-CD74 axis drives colorectal cancer via glycolytic reprogramming and is targeted by a novel small-molecule inhibitor

Jinwei Lou^{1,2} · Yuhan Chen^{1,2} · Yue Li¹ · Zixuan Liu¹ · Shangshang Hu^{2,3} · Muzi Ding^{1,2} · Jian Qin² · Huiling Sun^{1,2,4} · Yuqin Pan^{1,2,4}

Received: 27 September 2025 / Accepted: 25 March 2026
© The Author(s) 2026

Abstract

Background Macrophage migration inhibitory factor (MIF) promotes inflammation, regulates immune responses and chemotherapy resistance in the tumor microenvironment. However, its mechanism of action in colorectal cancer (CRC) metabolic reprogramming and targeted therapeutic potential remain unclear. This study aims to investigate the function, mechanism, and targeted therapeutic potential of MIF in CRC.

Methods Data were integrated from TCGA, GTEx, CPTAC, and HPA databases with clinical sample validation. Single-cell sequencing analysis (datasets GSE166555 and GSE144735) was performed, alongside functional assays and mechanistic studies. A novel high-potency MIF inhibitor was identified through virtual screening and validated in vitro and in vivo.

Results MIF expression was found to be significantly elevated in CRC tissues and cell lines, correlating with poor overall survival (OS) and disease-specific survival (DSS). Single-cell sequencing confirmed malignant epithelial cells as the primary MIF source. Functional assays demonstrated that MIF knockout suppressed CRC cell proliferation, migration, and tumor growth in vivo, while MIF overexpression promoted these effects. Mechanistically, MIF binds CD74 to upregulate glycolytic enzymes (HK2, PKM2, LDHA), enhancing glucose uptake and lactate/pyruvate production, thereby driving the Warburg effect and CRC progression. Virtual screening identified a novel high-potency MIF inhibitor, F3277-0933 (IC₅₀=8.284 μM). In vitro and in vivo, F3277-0933 surpassed the classical inhibitor ISO-1 in suppressing MIF-driven glycolytic reprogramming and proliferation.

Conclusion This study elucidates a novel mechanism by which the MIF-CD74 axis drives CRC progression through glycolytic reprogramming and provides robust preclinical evidence for developing MIF-targeted therapies.

Jinwei Lou and Yuhan Chen contributed equally to this work and should be considered co-first author.

✉ Huiling Sun
sunhuiling1988@yeah.net

✉ Yuqin Pan
panyuqin@njmu.edu.cn

¹ General Clinical Research Center, Nanjing First Hospital, China Pharmaceutical University, Nanjing, Jiangsu 211122, China

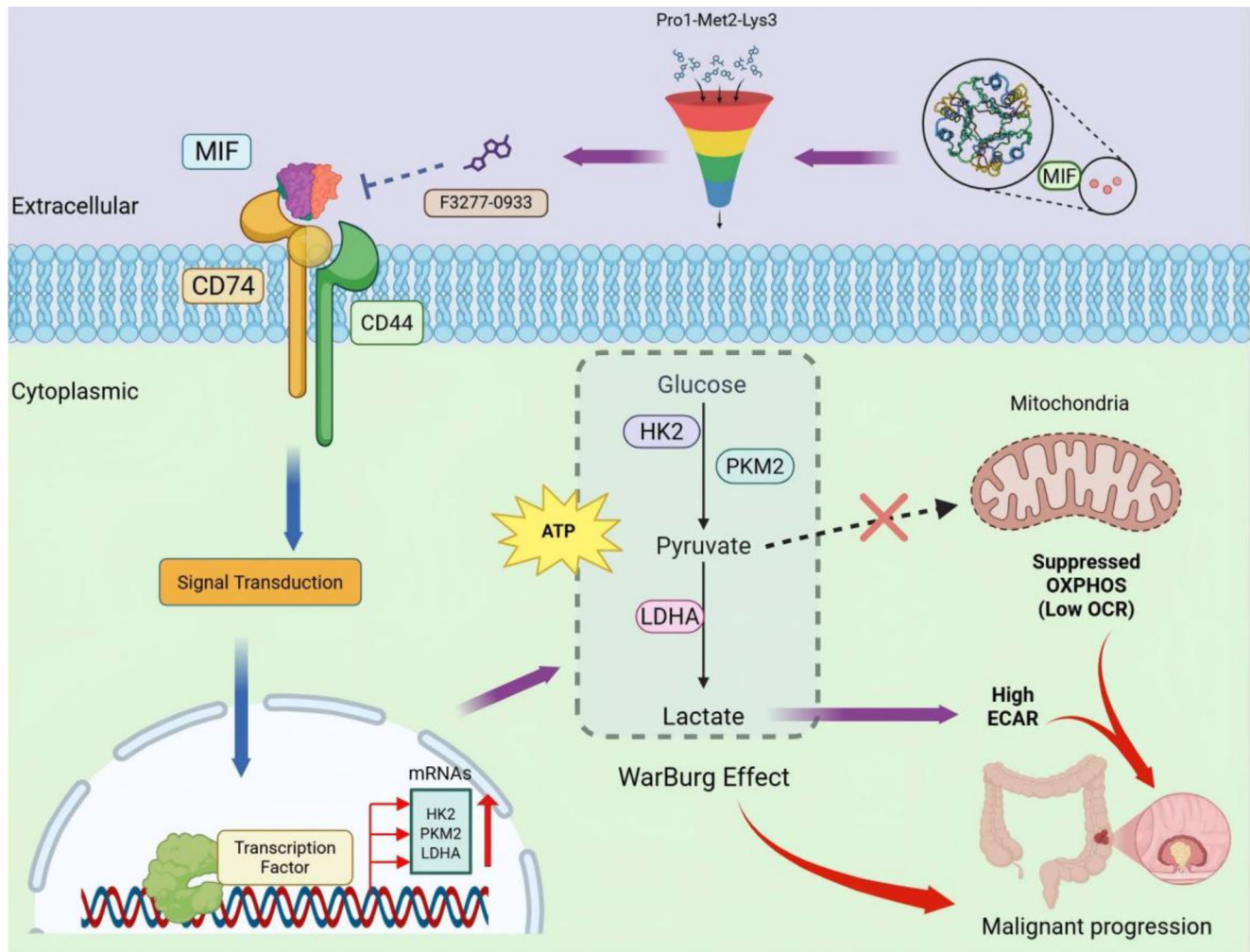
² General Clinical Research Center, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu 210006, China

³ School of Medicine, Southeast University, Nanjing, Jiangsu 210009, China

⁴ Jiangsu Collaborative Innovation Center on Cancer Personalized Medicine, Nanjing Medical University, Nanjing, Jiangsu 211100, China

Graphical Abstract

The MIF-CD74 axis drives colorectal cancer progression via glycolytic reprogramming. Extracellular MIF binds to the CD74/CD44 receptor complex on the surface of colorectal cancer cells, initiating downstream signaling cascades. This signal transduction leads to the transcriptional upregulation of key glycolytic enzymes (HK2, PKM2, and LDHA), driving a metabolic switch towards the Warburg effect—characterized by enhanced glucose uptake, increased lactate production (High ECAR), and suppressed mitochondrial oxidative phosphorylation (Low OCR). This metabolic reprogramming fuels malignant progression. The novel small-molecule inhibitor, F3277-0933, identified via structure-based virtual screening, specifically targets the MIF tautomerase active site. By blocking this oncogenic signaling axis, F3277-0933 effectively reverses the glycolytic phenotype and suppresses tumor growth.



Keywords Macrophage migration inhibitory factor (MIF) · Colorectal cancer · Glycolytic reprogramming · CD74 receptor · Small-molecule inhibitor · F3277-0933

1 Introduction

The global epidemiological burden of colorectal cancer (CRC) remains severe. By 2025, it is projected to rank third in new cancer cases and fourth in mortality, with an increasing incidence among younger populations. Regions such as China exhibit a significant acceleration in incidence rates

[1]. Conventional treatment centers on surgical resection, supplemented with fluorouracil/oxaliplatin-based chemotherapy, targeted therapy (e.g., anti-EGFR monoclonal antibodies for RAS wild-type patients), and immunotherapy (for microsatellite instability-high patients) [2]. However, current therapeutic regimens face significant limitations: over 50% of metastatic CRC patients experience treatment

failure due to primary or acquired resistance. Furthermore, patients with advanced disease exhibit poor response rates to interventions targeting liver or lung metastases, resulting in a 5-year survival rate below 15%. Consequently, urgent priorities include identifying novel therapeutic targets, developing specific inhibitors, and establishing a multi-omics-based classification system to guide precision treatment [3, 4].

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that exerts central regulatory effects on inflammation and tumorigenesis through its unique tautomerase enzymatic activity and interaction with the CD74 receptor [5–7]. Structurally, given that CD74 lacks an intracellular signaling domain, it recruits CD44 as an obligate co-receptor upon MIF binding. The assembly of this MIF-CD74-CD44 complex initiates downstream signaling cascades, particularly ERK1/2 and AKT phosphorylation, which synergistically drive tumor proliferation and metabolic reprogramming [8, 9]. In the tumor microenvironment, MIF promotes disease progression through multiple mechanisms: On one hand, it activates the CXCR4/CXCR7-CD74 signaling axis, drives JNK and MAPK/ERK pathway activation [10], induces the formation of an immunosuppressive microenvironment—such as inhibiting anti-tumor T-cell infiltration, promoting regulatory T-cell expansion and PD-L1 expression—thereby mediating immune evasion and immunotherapy resistance in tumors like melanoma and head and neck squamous cell carcinoma [11]; On the other hand, MIF enhances colorectal cancer chemotherapy resistance by upregulating CXCR7 [12], and promotes tumor metastasis by stabilizing β -catenin signaling. Notably, MIF secreted by breast cancer stem cells can reprogram tumor metabolism (e.g., lactate accumulation), directly suppressing immune cell function [13], while the MIF-ACKR3 pathway inhibits PPAR γ -mediated lipogenesis, leading to irreversible fat depletion associated with cancer cachexia [14]. In the field of inflammation, MIF not only drives inflammatory responses in sepsis [15], but also participates in the pathological processes of allergic diseases (e.g., asthma) by regulating eosinophil chemotaxis and activation [16]. Based on the above mechanisms, targeting MIF has become an important therapeutic strategy [17, 18]: The small-molecule inhibitor ISO-1, as a targeted drug, significantly improves sepsis survival by blocking tautomerase activity [19, 20]; Antagonists targeting MIF-CD74 interactions and covalent enzyme activity inhibitors (e.g., isothiocyanate derivatives) show potential to overcome tumor resistance in preclinical models [21]. These studies highlight the therapeutic value of MIF as a regulatory node.

The Warburg effect describes the phenomenon where cancer cells preferentially produce ATP through glycolysis rather than oxidative phosphorylation even under

aerobic conditions. A century after Otto Warburg's pioneering observation, it remains a cornerstone of cancer metabolism research [22]. Current research has elucidated the complex molecular mechanisms driving this metabolic reprogramming, identifying key pathways such as the PI3K/Akt axis as core regulators in promoting glycolytic flux and establishing environments supporting cancer cell proliferation and survival [23]. This metabolic shift confers significant selective advantages to cancer cells; It is now recognized that the Warburg effect supports aggressive phenotypes, induces therapy resistance, and drives tumor progression and metastasis by enhancing antioxidant defenses, nucleotide synthesis, and biomass accumulation [24]. Such metabolic patterns are particularly prominent in highly aggressive malignancies like triple-negative breast cancer [25]. Furthermore, contemporary studies reveal its profound immunological impact: The Warburg effect actively remodels the tumor microenvironment by depleting nutrients, generating lactate, and altering pH, thereby suppressing anti-tumor immune responses and promoting immune escape, marking it as a key regulator of tumor immunology [26]. In summary, despite a century having passed since Warburg's foundational discovery of cancer metabolic abnormalities, its far-reaching influence continues to shape modern oncology research and provides critical guidance for therapeutic strategies targeting metabolic vulnerabilities [27].

This study aims to investigate the function, mechanism, and targeted therapeutic potential of macrophage migration inhibitory factor (MIF) in colorectal cancer (CRC). Through integrated multi-database analysis and clinical sample validation, clarify the expression characteristics and prognostic value of MIF in CRC; Based on scRNA-seq, analyze the source and expression profile characteristics of MIF; Using functional experiments and mechanistic studies, reveal the molecular mechanism by which MIF regulates key glycolytic enzymes through the CD74 receptor to drive the Warburg effect; And identify novel high-activity MIF inhibitors through virtual screening, providing new mechanisms and candidate drugs for precision CRC therapy.

2 Results

2.1 MIF is aberrantly overexpressed in CRC and Predicts poor prognosis

Integrated analysis of TCGA and GTEx databases demonstrated significantly higher MIF mRNA expression in colorectal cancer (CRC) tissues compared to normal tissues (Fig. 1A–C). Survival analysis further revealed that patients with high MIF expression had significantly shorter overall survival (Stage I and II) (OS) and disease-specific survival

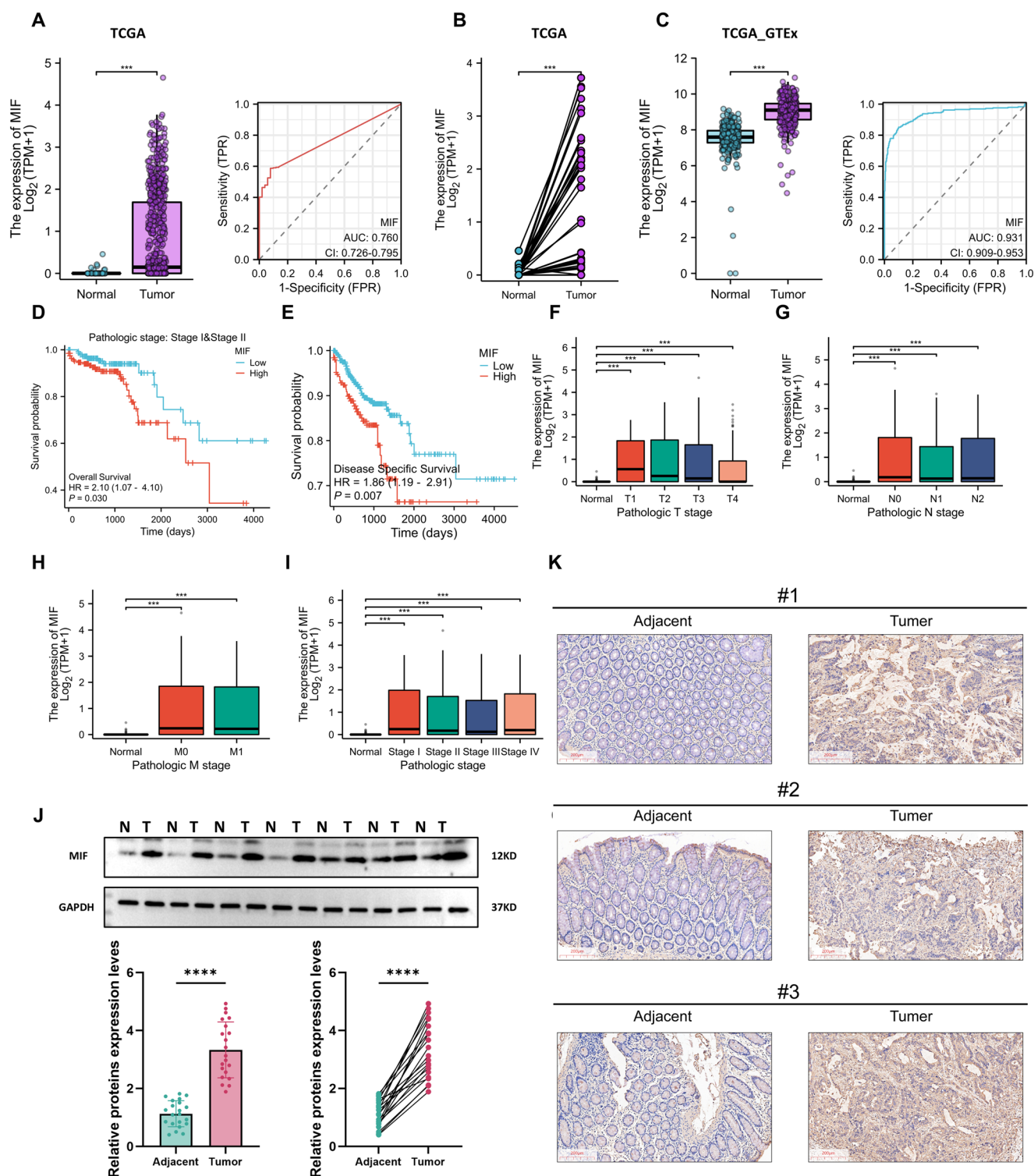


Fig. 1 MIF is Aberrantly Overexpressed in CRC and Predicts Poor Prognosis. **(A)** MIF expression levels in normal versus tumor tissues and the receiver operating characteristic (ROC) curve based on the TCGA database. **(B)** Paired difference analysis of MIF expression between normal and tumor tissues in the TCGA database. **(C)** MIF expression levels in normal versus tumor tissues and the ROC curve based on the TCGA-GTEx Combined Database. **(D-E)** Kaplan-Meier survival analysis showing the correlation between high/low

MIF expression and overall survival (OS) or disease-specific survival (DSS) in patients with Stage I and II CRC. **(F-I)** Comparison of MIF expression across different pathological TNM stages. **(J)** Representative Western blot images and quantitative analysis of MIF protein expression in 21 paired CRC tumor tissues (T) and adjacent normal tissues (N). **(K)** Representative immunohistochemical (IHC) staining images validating the spatial expression and intensity of MIF in CRC clinical specimens (Scale bar = 200 μm)

(DSS), indicating MIF as a prognostic risk factor for CRC (Fig. 1D–E). Across CRC samples of different TNM stages, MIF expression consistently showed an upregulated trend compared to normal tissues (Fig. 1F–I). Notably, MIF expression peaked in early-stage (Stage I/II) samples, suggesting its upregulation is an early, critical event in colorectal carcinogenesis that drives the initial metabolic switch. Proteomic analysis from the CPTAC database also revealed significantly higher MIF expression levels in CRC tissues versus normal tissues (Supplementary Figure 1A). Immunohistochemistry (IHC) results from the HPA database further confirmed strong positive staining for MIF in CRC (Supplementary Figure 1B–G). To strengthen clinical relevance, this study collected 21 paired tumor/adjacent tissue samples from CRC patients for validation. Western blot analysis demonstrated significantly upregulated MIF protein expression in tumor tissues compared to adjacent tissues (Fig. 1J). IHC results similarly showed specific high expression of MIF in tumor regions (Fig. 1K).

2.2 MIF primarily originates from malignant tumor cells

This study integrated two single-cell datasets (GSE166555 and GSE144735), comprising 37 samples (19 tumor, 18 normal tissues). After batch correction and quality control filtering, 82,865 cells were retained. Subsequent clustering analysis showed no significant batch effects across samples, stages, or groups in t-SNE plots (Fig. 2A). Based on cell surface-specific marker genes (Supplementary Figure 2), we identified seven major cell types (Fig. 2B).

To clarify MIF expression patterns, we performed clustering analysis comparing tumor and non-tumor tissue samples. Results revealed significantly higher MIF expression in tumor tissues versus normal tissues (Fig. 2C–D). Given MIF's particularly prominent expression in epithelial cells (Fig. 2E–F), we focused on epithelial cell populations, conducting clustering analysis of epithelial cells derived from tumor and non-tumor tissues. Results showed significantly elevated MIF expression in tumor-derived epithelial cells (Fig. 2G–I). For precise localization of high MIF-expressing subpopulations, we distinguished malignant from non-malignant epithelial cells within tumor-derived epithelial populations (Fig. 2J). Notably, MIF expression was significantly upregulated in malignant epithelial cells compared to non-malignant counterparts (Fig. 2K–L). Therefore, MIF primarily originates from malignant tumor cells.

Using human normal intestinal epithelial cells (NCM460) and CRC cell lines (HCT-116, HCT-8, HCT-15, HT-29, LOVO, DLD1, SW620, SW480), we extracted proteins and RNA. RT-qPCR and Western blot analyses revealed significantly upregulated MIF expression in CRC cell lines versus

NCM460 normal intestinal epithelial cells. LOVO cells showed the highest expression level, while DLD1 exhibited the lowest (Supplementary Figure 3A–B). Based on this expression profile, we successfully constructed stable MIF-knockout LOVO cells and MIF-overexpressing DLD1 cells, with knockout/overexpression efficiency validated by Western blot (Fig. 3A–B). Among the three sgRNAs tested, sgRNA-2 showed the most potent knockout effect (Fig. 3A) and was used for all subsequent experiments (hereafter referred to as sgMIF).

2.3 MIF promotes CRC cell proliferation and migration

To investigate MIF's biological function in colorectal cancer (CRC), we conducted *in vitro* and *in vivo* experiments. Colony formation assays revealed that MIF knockout significantly suppressed colony formation capacity in CRC cells (Fig. 3C), whereas MIF overexpression markedly enhanced this capability (Supplementary Figure 4A). EdU proliferation assays further validated these results: MIF deficiency inhibited cell proliferation (Fig. 3D), while its overexpression promoted proliferation (Supplementary Figure 4B). Additionally, Transwell migration and invasion assays consistently demonstrated that MIF knockout significantly reduced cell migratory capacity (Fig. 3E–F), whereas MIF overexpression substantially increased migratory ability (Supplementary Figure 4C–D). These results confirm that *in vitro* experiments demonstrate MIF's functional role in promoting CRC cell proliferation and migration.

Using these cell lines, we established subcutaneous xenograft models in nude mice. Results showed significantly smaller tumor volumes and weights in the MIF-knockout group compared to controls (Fig. 3G–I), while the overexpression group exhibited significant increases (Supplementary Figure 4 E–G). Histological analysis revealed a significantly lower Ki-67-positive cell rate in MIF-knockout xenografts versus controls, with H&E staining indicating reduced tumor malignancy (Fig. 3J). Conversely, the overexpression group showed significantly elevated Ki-67 positivity and malignancy (Supplementary Figure 4H). Collectively, these *in vitro* and *in vivo* findings indicate that MIF drives CRC progression by enhancing tumor cell proliferation and motility.

2.4 MIF drives CRC progression by remodeling glycolytic metabolism

To elucidate the molecular mechanism by which MIF promotes colorectal cancer (CRC) progression, we stratified TCGA database samples into high- and low-MIF expression

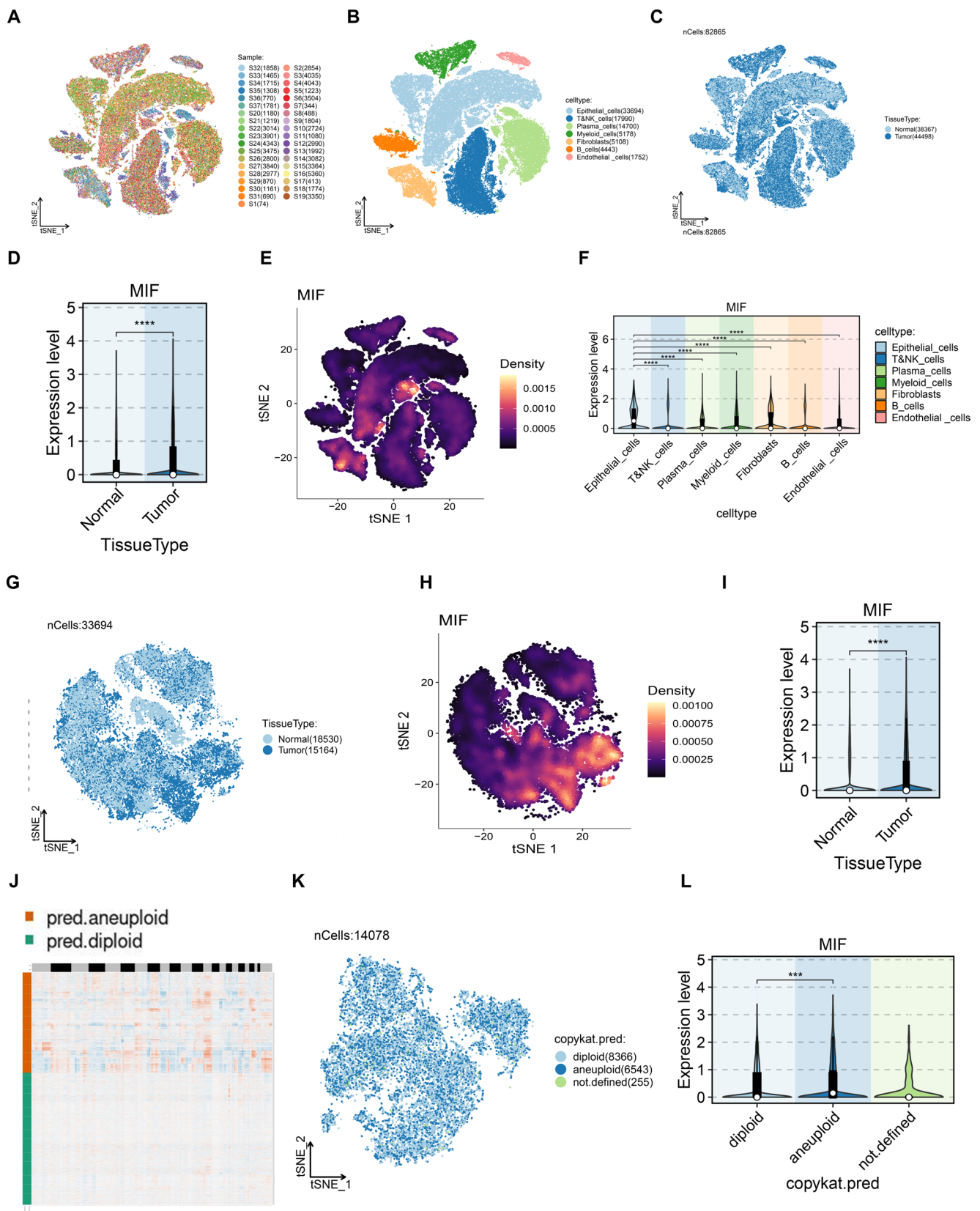


Fig. 2 Single-cell transcriptomic profiling identifies malignant epithelial cells as the primary source of MIF. **(A)** t-SNE visualization colored by sample origin. The dataset includes 37 samples (Tumor: S1–S19; Normal: S20–S37). Batch effects were corrected using Harmony to ensure clustering by biological identity rather than patient origin. **(B)** t-SNE visualization of cell lineages. The plot displays the distribution of the seven major cell subpopulations identified across the integrated tumor and normal datasets. **(C–D)** Comparative analysis of MIF expression between tumor and normal tissues. The t-SNE feature plot **(C)** and quantitative violin plot **(D)** collectively illustrate that MIF expression is significantly upregulated in tumor samples compared to normal controls. **(E–F)** Cell-type specific expression of MIF. The feature plot **(E)** displays the global distribution of MIF across all cell subpopulations, while the violin plot **(F)** quantifies expression levels across the seven major lineages. **(G–I)** Comparative analysis of MIF expression levels between normal intestinal epithelial cells and tumor-derived epithelial cells. **(G)** Sub-clustering visualization of isolated epithelial cells colored by sample origin. **(H–I)** Quantitative analysis demonstrating that MIF expression is significantly upregulated in tumor-derived epithelial cells compared to normal controls. **(J)** Identification of malignant epithelial cells using the “copykat” R package based on aneuploidy inference. **(K–L)** Targeted analysis of MIF expression levels specifically within the malignant epithelial cell population compared to other clusters. Statistical significance for differential expression was determined using the Wilcoxon rank-sum test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

groups, identifying differentially expressed genes (DEGs) for functional enrichment analysis (Fig. 4A). KEGG and GO analyses revealed significant enrichment of DEGs in glycolytic, pyruvate metabolism, and related metabolic pathways (Fig. 4B–E). Gene Set Variation Analysis (GSVA) and Gene Set Enrichment Analysis (GSEA) further confirmed stronger enrichment in glycolysis, mTORC1 signaling, and lipid metabolism pathways in high-MIF versus low-MIF groups (Fig. 4F–G). To clarify MIF’s association with tumor metabolic reprogramming, we subdivided malignant epithelial cell subpopulations into MIF⁺ and MIF[−] clusters (Fig. 4H). Warburg effect scores demonstrated significantly higher scores in MIF⁺ versus MIF[−] malignant cells (Fig. 4I–J). Hallmark and KEGG gene set enrichment analyses both indicated specific activation of glycolytic pathways in MIF⁺ malignant cells (Fig. 4K–L).

To confirm the regulatory role of MIF in metabolic reprogramming, we performed a systematic metabolic phenotype analysis in established MIF-knockout (LOVO) and MIF-overexpressing (DLD1) cell models. Western blot analysis revealed that MIF knockout significantly downregulated the protein expression of key glycolytic enzymes (HK2, PKM2, and LDHA), whereas MIF overexpression upregulated them (Fig. 5A–B). Metabolically, MIF knockout significantly reduced glucose uptake and decreased lactate and pyruvate production, while the overexpression group showed the opposite trend (Fig. 5C–H). To further dissect the real-time impact of MIF on mitochondrial respiration and glycolytic function, we conducted Seahorse metabolic flux analysis. The results demonstrated that MIF knockout significantly

increased maximal respiration (OCR) while decreasing glycolytic capacity (ECAR) (Fig. 5I–J). Conversely, MIF overexpression significantly suppressed OCR and elevated ECAR (Fig. 5K–L). Collectively, these findings indicate that MIF potentially drives the Warburg effect in CRC cells by upregulating key glycolytic enzymes and concurrently inhibiting mitochondrial oxidative phosphorylation.

2.5 CD74 mediates MIF-Induced glycolytic metabolic reprogramming in CRC

To elucidate the molecular mechanism by which MIF regulates the Warburg effect, we analyzed intercellular communication networks between MIF⁺ and MIF[−] malignant epithelial subpopulations. Results revealed that the MIF⁺ malignant cell subpopulation exhibited the strongest autocrine signaling (Fig. 6A–B). Similarly, when examining the MIF signaling pathway specifically, this subpopulation also displayed the most pronounced self-reinforcing signaling (Fig. 6C). Screening of differential ligand-receptor pairs identified MIF-CD74 as the sole autocrine pair in MIF⁺ malignant cells (Fig. 6D), indicating CD74 as a key downstream effector of MIF. Consistently, CD74 expression was significantly higher in MIF⁺ versus MIF[−] subpopulations (Fig. 6E). To clarify CD74’s role in MIF-mediated glycolytic reprogramming, we performed functional validation using the humanized anti-CD74 monoclonal antibody (Milatuzumab) and recombinant human MIF (rhMIF). In CD74 blockade experiments, Milatuzumab significantly downregulated key glycolytic proteins (HK2, PKM2, LDHA) while simultaneously reducing glucose uptake, lactate production, and pyruvate output. In rescue experiments, adding exogenous rhMIF failed to reverse the suppressed expression of glycolytic proteins or the decline in key metabolite levels even under Milatuzumab-mediated CD74 blockade (Fig. 6F–I). These findings demonstrate that CD74 is an essential receptor for MIF-induced activation of the Warburg effect. Blocking CD74 effectively suppresses MIF’s ability to promote glycolysis.

2.6 Structure-based virtual screening of MIF-Targeted small-molecule inhibitors and antitumor activity Validation

This study employed computer-aided drug design strategies to screen MIF-targeted small-molecule inhibitors (Fig. 7A). First, Schrödinger software performed SP-XP multistage virtual screening on the LF1000 library (using MIF protein as target), initially obtaining 3,271 binding conformations and retaining 2,331 optimal conformation molecules (Fig. 7B). Key binding site analysis identified ILE64 as the core interaction site (1,595 molecules interacting) with PRO1 as

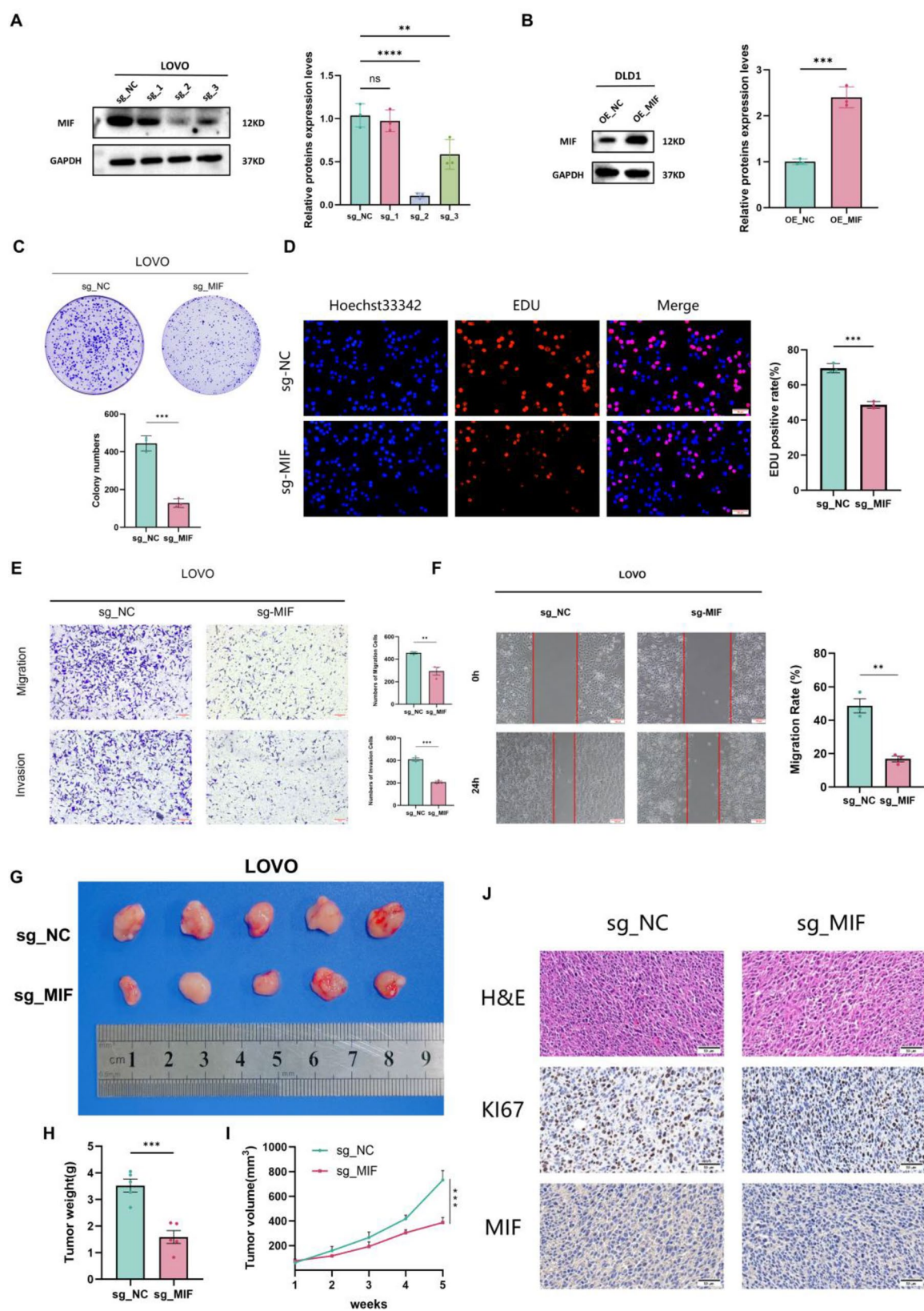


Fig. 3 MIF promotes CRC cell proliferation, migration, and invasive capacity. (A–B) Validation of knockout/overexpression efficiency via Western blot analysis. (A) Confirmation of MIF knockout efficacy in LOVO cells (sg_MIF vs. sg_NC). (B) Confirmation of MIF overexpression efficacy in DLD1 cells (OE_MIF vs. OE_NC). (C) Representative images and statistical analysis of colony formation assays assessing the long-term proliferative capacity of MIF-knockout cells. (D) EdU incorporation assays evaluating DNA synthesis activity. Red fluorescence indicates EdU-positive proliferating cells; blue fluorescence indicates nuclei stained with Hoechst 33,342 (Scale bar=50 μ m). (E–F) Assessment of cell motility. Transwell migration and invasion assays (E, Scale bar=10 μ m) and wound healing assays (F, Scale bar=100 μ m) demonstrate significant impairment of migratory and invasive capabilities in MIF-knockout cells. (G–I) Evaluation of tumorigenesis in a nude mouse xenograft model ($n=5$ per group). Representative images of excised tumors (G), tumor weight statistics (H), and tumor volume growth curves (I). (J) Representative H&E staining and immunohistochemical (IHC) analysis of Ki67 and MIF expression in xenograft tissues (Scale bar=50 μ m). For all box plots, the center line represents the median, the bounds of the box represent the 25th and 75th percentiles, and the whiskers represent the minimum and maximum values. Data are presented as mean $\pm$ SD ($n=3$ independent experiments). Statistical significance was determined using the Log-rank test (D–E), paired Student's *t*-test (B, J; $n=21$ paired samples for J), one-way ANOVA followed by Tukey's post-hoc test (F–I), and unpaired Student's *t*-test or Wilcoxon rank-sum test (A, C). Exact *p*-values are detailed in Supplementary Table S1. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

secondary (Fig. 7C). After excluding 172 non-interacting molecules, 2,159 high-potential candidates were obtained. Molecules with binding energies <-80 kcal/mol were further screened via MM/GBSA calculation. Following protein-ligand interaction fingerprint (PLIF) filtering and MOE software clustering (70% structural similarity threshold), optimal representatives from 308 structurally diverse clusters were retained.

Given MIF's tautomerase activity mechanism, this study evaluated inhibitor efficacy against this enzymatic function using L-dopachrome methyl ester as the substrate. The top 30 compounds by molecular docking score were selected for tautomerase activity testing, with the prototype MIF inhibitor ISO-1 ($IC_{50}=14.14 \mu$ M) as positive control. Results revealed seven compounds with significantly stronger inhibitory activity (i.e., lower IC_{50} values) than ISO-1. Among these, the small-molecule inhibitor F2881-0530 showed the highest potency ($IC_{50}=1.910 \mu$ M), while compound F1736-2062 exhibited the weakest activity ($IC_{50}=12.29 \mu$ M) (Fig. 7D). The inhibition curves for the remaining 23 compounds are included in the supplementary materials (Supplementary Figure 5A).

To evaluate the anti-tumor activity of inhibitors, we selected the seven compounds with superior tautomerase inhibition over ISO-1 for functional validation. When applied to colorectal cancer (CRC) LOVO cells at gradient concentrations (0–100 μ M), three compounds—F3371-0690 ($IC_{50}=19.86 \mu$ M), F3277-0933 ($IC_{50}=8.284 \mu$ M), and F2881-0530 ($IC_{50}=66.96 \mu$ M)—exhibited dose-dependent

inhibition of cell proliferation within this concentration range (Fig. 7E, Supplementary Figure 5B). Molecular docking simulations further revealed that F3277-0933 and other candidates form stable hydrogen bonds and hydrophobic interactions with key residues (e.g., Pro1, Lys32, Ile64) within the MIF active pocket (Fig. 7F–H, Supplementary Figure 5C). Notably, both F3371-0690 and F3277-0933 demonstrated superior suppression of LOVO cell proliferation compared to the control compound ISO-1 ($IC_{50}=38.89 \mu$ M). In summary, the small-molecule inhibitor F3277-0933 demonstrated highly potent inhibitory activity against MIF tautomerase while also showing significantly greater potential than other candidate molecules in anti-tumor activity screening in vitro. Therefore, we selected F3277-0933 for further in-depth in vitro and in vivo studies.

2.7 The novel MIF inhibitor F3277-0933 inhibits CRC cells proliferation and glycolytic metabolism

To systematically evaluate the anti-tumor effects of the novel MIF-targeting inhibitor F3277-0933, this study employed multi-level experimental models and compared it with the control inhibitor ISO-1. In in vitro models, treatment of LOVO cells with F3277-0933 demonstrated significantly superior inhibition of cell proliferation compared to ISO-1, as shown by both colony formation and EdU assays (Fig. 8A, B). Concurrently, F3277-0933 more significantly downregulated the expression of key glycolysis-related proteins (HK2, PKM2, LDHA) than ISO-1, and more potently inhibited glucose uptake, as well as lactate and pyruvate production (Fig. 8C–F). To validate its real-time impact on metabolic flux, Seahorse analysis demonstrated that F3277-0933 treatment significantly increased the oxygen consumption rate (OCR) and decreased the extracellular acidification rate (ECAR) in CRC cells compared to ISO-1 (Supplementary Figure 6A). This indicates that F3277-0933 effectively reverses the Warburg effect by suppressing glycolysis and restoring mitochondrial oxidative phosphorylation. In a more clinically relevant context, we validated these findings using two independent patient-derived organoid (PDO) lines. F3277-0933 treatment of CRC patient-derived organoids for 6 days resulted in more significant volume reduction and a higher proportion of apoptotic cells as assessed by Calcein-AM/PI double staining

(Fig. 8G, Supplementary Figure 6B). In in vivo xenograft models, groups treated with F3277-0933 (100 mg/kg, three times weekly for four weeks) (Fig. 8H) showed significantly greater suppression of tumor volume and weight compared to those receiving ISO-1 (Fig. 8I–K). Histological analysis further revealed the lowest MIF and Ki-67 positive rate, with H&E staining indicating reduced tumor malignancy

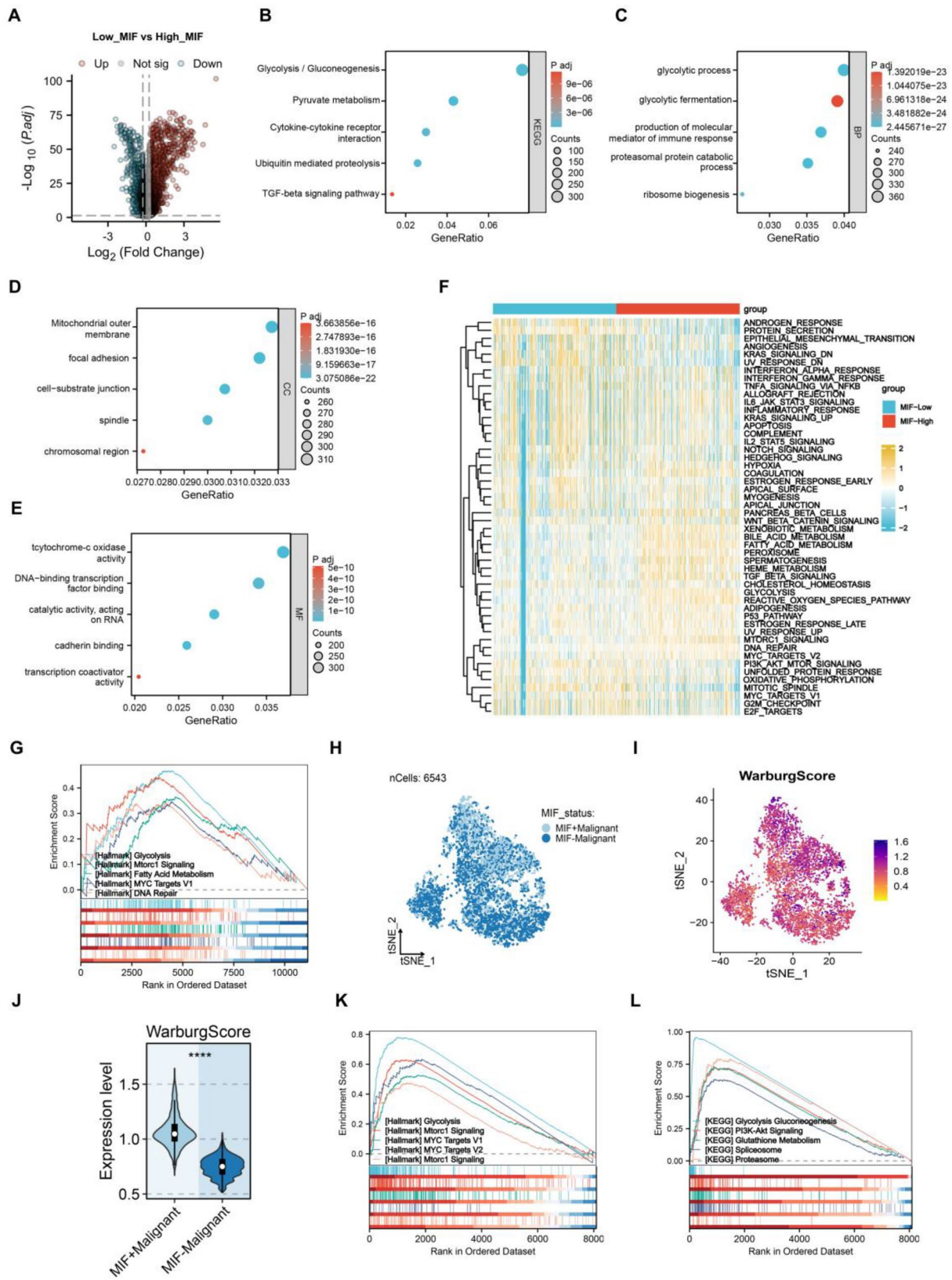


Fig. 4 MIF Drives CRC Progression by Remodeling Glycolytic Metabolism. (A) Differential gene screening between MIF-high and MIF-low expression groups in TCGA cohort. (B–E) KEGG pathway enrichment profiles and GO functional annotation of MIF-related differential genes. (F–G) Gene Set Variation Analysis (GSVA enrichment scores) and Gene Set Enrichment Analysis (GSEA) in MIF-high/low expression groups. (H) UMAP visualization of malignant epithelial cells isolated via a sequential strategy (Epithelial → Malignant [CopyKAT] → MIF⁺/MIF[−]). (I–J) Quantitative comparison of Warburg effect scores in MIF⁺/MIF[−] malignant epithelial cell subpopulations. (K–L) GSEA enrichment profiles of hallmark/KEGG gene sets for MIF⁺ malignant cell signature genes. Data are presented as mean ± SD. Statistical significance was determined using the Wilcoxon test. *****p* < 0.0001

(Fig. 8L). Collectively, F3277-0933, inhibiting tumor proliferation and the glycolytic metabolism, demonstrated comprehensively superior anti-CRC activity over ISO-1 in preclinical models.

3 Discussion

This study systematically elucidates the oncogenic role of MIF in colorectal cancer (CRC) and its molecular mechanisms, while exploring MIF-targeted therapeutic strategies. We confirmed MIF overexpression in CRC and its role as a poor prognostic factor. More importantly, through in-depth functional and mechanistic investigations, we reveal for the first time that MIF specifically activates the Warburg effect (glycolytic metabolic reprogramming) in cancer cells via its receptor CD74-mediated signaling, thereby driving CRC proliferation and migration. This discovery extends the known pro-inflammatory and immunomodulatory functions of MIF in the tumor microenvironment (TME) [28] to tumor cell-autonomous metabolic regulation, providing a novel metabolic dimension for understanding MIF-driven tumor progression [29].

Metabolic reprogramming enables tumor cells to reshape energy and biosynthetic pathways to support proliferation. By altering key metabolic processes (e.g., glucose, lipid, and glutamine metabolism), it drives cellular state transitions, gene expression reprogramming, and microenvironment remodeling, ultimately promoting tumor progression and therapy resistance [30]. Dysregulated glucose metabolism is central to this process: tumor cells preferentially utilize aerobic glycolysis over mitochondrial oxidative phosphorylation (the Warburg effect), generating lactate and biosynthetic precursors to fuel rapid proliferation. This hallmark metabolic feature is recognized as one of cancer's ten defining characteristics [31]. Our study demonstrates that MIF directly upregulates key glycolytic enzymes (HK2, PKM2, LDHA) through the CD74 receptor-mediated autocrine axis, driving the Warburg effect to accelerate CRC progression.

Existing studies report HK2, LDHA, and PKM2 overexpression in therapy-resistant tumors (e.g., liver, breast,

esophageal cancers), closely linked to chemo- and targeted therapy resistance [32]. Elevated glycolytic flux in resistant tumors increases lactate accumulation, which lactylates DNA repair protein NBS1 and impairs chemotherapeutic efficacy (e.g., cisplatin) [33]. TP53 mutation activates deubiquitinase UCHL3 via JAK2–STAT3 signaling, stabilizing glycolytic enzyme ENO1 to promote 5-FU resistance in CRC [34]. Extracellular vesicles (EVs) from resistant cells carry metabolic proteins (e.g., GSTP1) and ncRNAs (e.g., miR-21-5p, circZNF91), reprogramming glycolysis and lipid metabolism in recipient cells to transmit resistance [35]. Our study identifies specific glycolytic pathway activation in MIF-high CRC patients with poorer prognosis, suggesting that targeting the MIF-CD74-glycolysis axis may reverse therapy resistance in this subset.

Our study highlights the critical role of MIF in regulating metabolic plasticity in colorectal cancer. A critical aspect of tumor survival is metabolic plasticity—the capacity of tumor cells to dynamically switch between glycolysis and oxidative phosphorylation (OXPHOS) to adapt to metabolic stress. Specifically, our Seahorse metabolic flux analysis revealed that while MIF-overexpressing cells are locked in a hyper-glycolytic state (high ECAR), MIF-silenced cells exhibit a compensatory shift towards mitochondrial respiration. This identifies MIF as a pivotal ‘metabolic switch’ that forces cancer cells into the Warburg phenotype. Consequently, targeting MIF with F3277-0933 not only suppresses glycolysis but also disrupts this metabolic flexibility, effectively destabilizing the energetic adaptability required for aggressive growth and invasion. Furthermore, our single-cell RNA sequencing analysis provides a granular view of the metabolic heterogeneity within CRC tumors. We observed that not all malignant cells uniformly express high levels of MIF; rather, a distinct MIF-high subpopulation co-exists alongside MIF-low clusters. This observation supports the concept of ‘metabolic mosaicism,’ where different tumor cell populations adapt to specific microenvironmental niches. We propose that MIF-high cells likely function as the ‘glycolytic engines’ of the tumor, driving rapid proliferation and invasion via the Warburg effect, potentially residing in hypoxic regions. Conversely, co-existing MIF-low populations may rely on alternative metabolic pathways (such as oxidative phosphorylation) or remain in a quiescent state to survive nutrient deprivation. This metabolic heterogeneity represents a critical survival strategy, allowing the tumor ecosystem to thrive under fluctuating environmental conditions and highlighting the importance of targeting the dominant glycolytic subpopulation via the MIF-CD74 axis.

A key breakthrough is establishing CD74 as the indispensable receptor for MIF-induced glycolytic reprogramming. Blocking CD74 with the specific antibody Milatuzumab inhibited MIF-driven upregulation of glycolytic enzymes

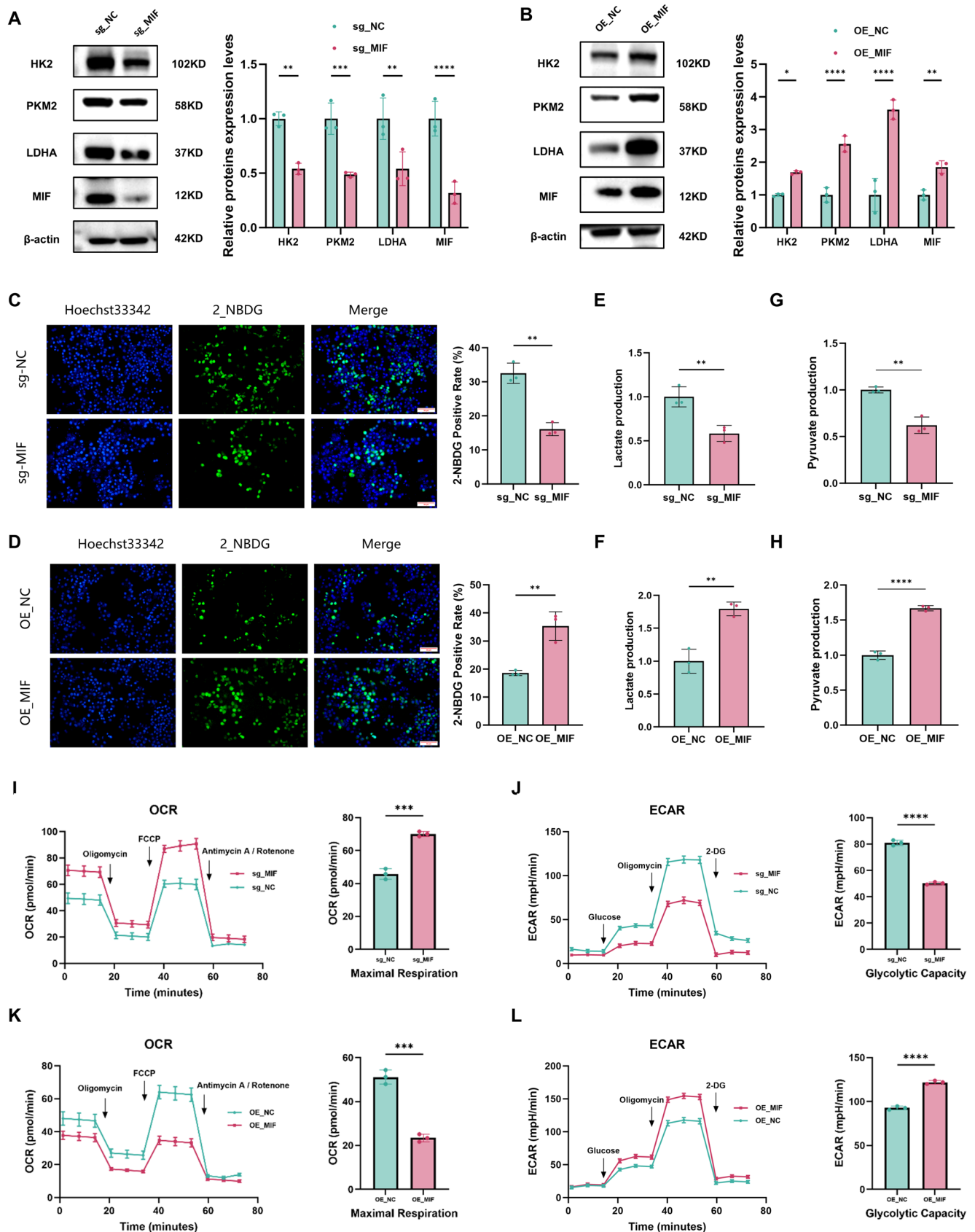


Fig. 5 Metabolic Phenotype Detection in MIF-Knockout (LOVO) and MIF-Overexpressing (DLD1) Cell Models (A–B) Regulatory effect of MIF knockout/overexpression on Warburg effect key proteins (HK2, LDHA, PKM2) expression (Western blot analysis). (C–D) Effect of MIF knockout/overexpression on glucose uptake capacity in CRC cells (Glucose uptake assay; Scale bar=50 μ m). (E–F) Effect of MIF knockout/overexpression on lactate production in CRC cells (Lactate content detection). (G–H) Effect of MIF knockout/overexpression on pyruvate production in CRC cells (Pyruvate content detection). (I) Impact of MIF knockout (sg_MIF) on cell OCR and statistical analysis of maximal respiration. (J) Impact of MIF knockout (sg_MIF) on cell ECAR and statistical analysis of glycolytic capacity. (K) Impact of MIF overexpression (OE_MIF) on cell OCR and statistical analysis of maximal respiration. (L) Impact of MIF overexpression (OE_MIF) on cell ECAR and statistical analysis of glycolytic capacity. Data are presented as mean $\pm$ SD ($n=3$ independent experiments). Statistical significance was determined using the unpaired Student's *t*-test. Exact *p*-values are detailed in Supplementary Table S1. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

and metabolites (glucose uptake, lactate, pyruvate), which exogenous recombinant human MIF (rhMIF) failed to rescue. This confirms the centrality of the MIF-CD74 ligand-receptor pair in autocrine signaling within malignant epithelial cells (especially MIF-high subpopulations), providing direct mechanistic support for its therapeutic targeting. Single-cell data further reinforce the critical role of MIF⁺ malignant subpopulations in glycolytic activation and poor patient outcomes.

Virtual screening—a cornerstone of modern drug discovery—leverages core advantages (high throughput, low cost, expansive search capabilities) to play pivotal roles [36]. Structure-based virtual screening (SBVS) has proven to be a powerful strategy for accelerating drug discovery by efficiently narrowing down candidate compounds. In this study, the successful identification of F3277-0933 validates the reliability of our multistage screening workflow (combining SP/XP docking and MM/GBSA calculations) in targeting the MIF tautomerase active site. This approach allowed us to identify high-potency inhibitors with reduced time and cost compared to traditional high-throughput screening. Validated by structure-based screening and functional assays, F3277-0933 potently inhibited CRC cell proliferation in vitro and suppressed xenograft growth in vivo, outperforming the reference inhibitor ISO-1. Crucially, it reversed MIF-driven glycolytic reprogramming by downregulating key enzymes and reducing metabolite production. Significant anti-tumor activity was also observed in clinically relevant CRC patient-derived organoid (PDO) models. These results strongly support targeting MIF tautomerase activity as a novel strategy to overcome therapy resistance in CRC, particularly in subtypes reliant on the MIF-CD74-glycolysis axis.

Despite these advances, several limitations warrant consideration. First, the functional validation of MIF-CD74 axis dependency relied primarily on in vitro models and

immunodeficient mouse xenografts, which may not fully recapitulate the complexity of human tumor-microenvironment interactions, particularly immune cell-mediated metabolic crosstalk. Second, while virtual screening identified F3277-0933 as a potent inhibitor, its pharmacokinetic properties (e.g., bioavailability, metabolic stability) and potential off-target effects warrant further characterization. Third, the mechanistic link between MIF-CD74 signaling and glycolytic enzyme upregulation (e.g., direct transcriptional regulation vs. post-translational modifications) requires further elucidation. Lastly, clinical correlations were derived from retrospective databases; prospective validation in larger cohorts is needed to confirm MIF's utility as a biomarker for metabolic subtyping.

4 Conclusion

In summary, this study establishes that the MIF-CD74 axis drives colorectal cancer (CRC) progression via glycolytic metabolic reprogramming (the Warburg effect). We identified a highly potent MIF inhibitor, F3277-0933, through structure-based virtual screening, which demonstrated exceptional antitumor activity in preclinical models. These findings deepen our understanding of metabolic heterogeneity in CRC and the multifunctional role of MIF, providing a solid experimental foundation for the clinical translation of MIF-targeted precision therapy. Future research should prioritize validating MIF as a biomarker for metabolic subtyping in larger cohorts and evaluating the synergistic efficacy of F3277-0933 in combination with standard therapies (chemotherapy or immunotherapy), offering new avenues to improve outcomes for patients with refractory or metastatic CRC.

5 Materials and methods

5.1 Data collection and processing

This study integrated multi-center colorectal cancer (CRC) cohort data from published literature, The Cancer Genome Atlas (TCGA), and Gene Expression Omnibus (GEO) database. Single-cell transcriptomic data were sourced from GEO database, including GSE144735 [37] (PMID: 32451460) and GSE166555 [38] (PMID: 34409732). Single-cell transcriptomic data processing was based on Seurat R package. Quality control conditions: Cells retaining 200–5,000 detected genes with mitochondrial gene proportion <20% were retained; For single-cell transcriptomic data, gene expression matrices were normalized using the SCTransform method in Seurat to regress out variation

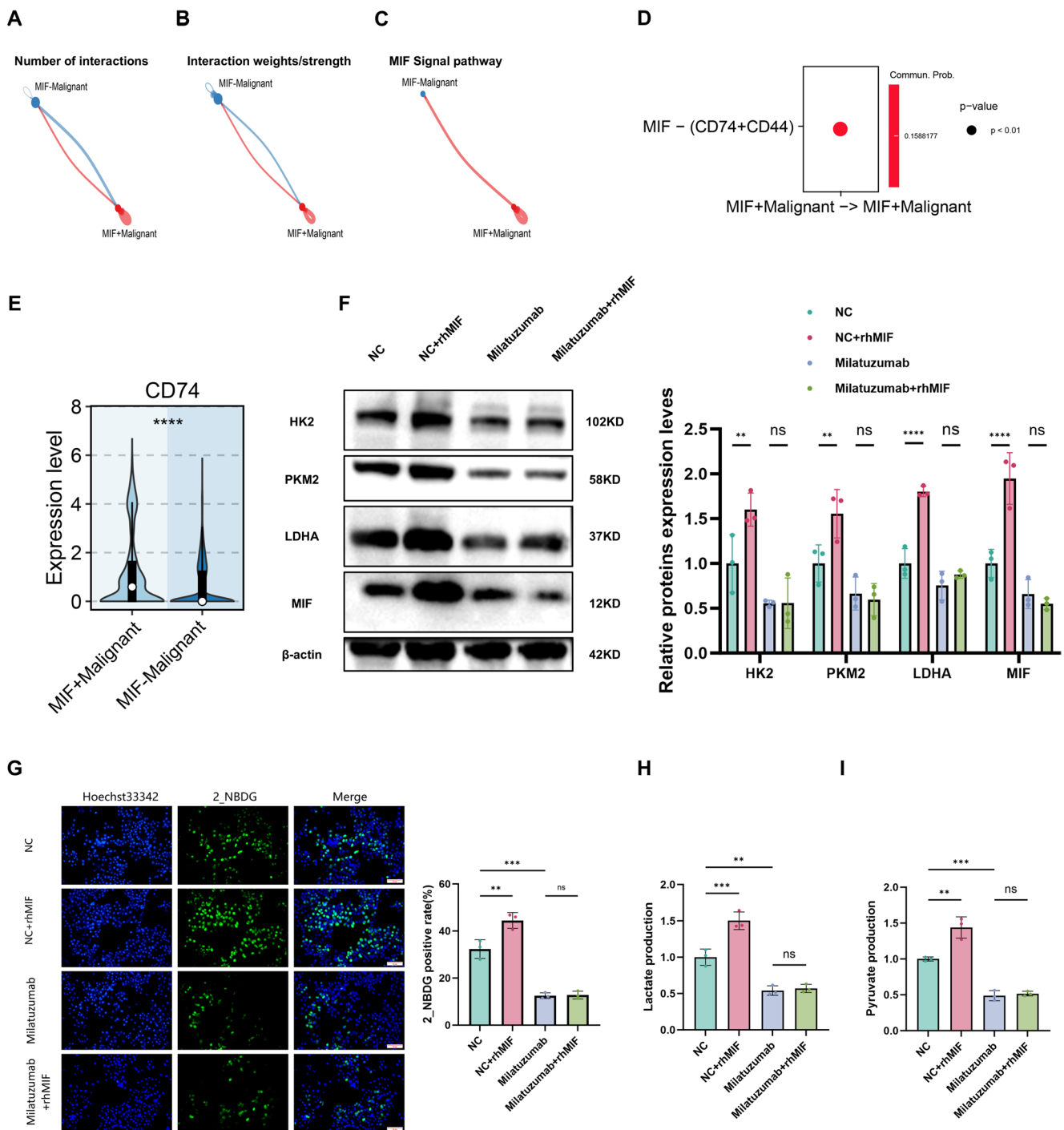


Fig. 6 CD74 Mediates MIF-Induced Glycolytic Metabolic Reprogramming in CRC. (A–C) CellChat visualization of intercellular communication. Circle plots display inferred interactions (lines) between cell clusters (nodes), with edge thickness proportional to interaction number (A) or strength (B–C). Specifically, (A–B) illustrate the aggregated interaction frequency and strength involving MIF⁺/MIF⁻ malignant epithelial cells, while (C) details local communication mediated specifically by the MIF signaling pathway. (D) Key ligand-receptor pairs mediating autocrine communication in MIF⁺ malignant epithelial cells. (E) Differential CD74 mRNA expression levels in MIF-positive (MIF⁺) versus MIF-negative (MIF⁻) malignant epithelial cell

subpopulations identified via single-cell RNA sequencing. (F) Regulatory effect of rhMIF/Milatuzumab treatment on key Warburg effect proteins (HK2, LDHA, PKM2) in LOVO cells (Western blotting). (G–I) Effects of rhMIF/Milatuzumab treatment on glucose uptake, lactic acid production, and pyruvic acid production in LOVO cells (G, Scale bar = 50 μ m). Data are presented as mean $\pm$ SD (n = 3 independent biological replicates). Statistical comparisons between multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. Exact p -values are detailed in Supplementary Table S1. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

due to sequencing depth, followed by batch effect elimination using the ‘Harmony’ package [39] (PMID:31740819) to ensure cells clustered by biological identity rather than patient origin. Bulk transcriptomic data came from TCGA, Genotype-Tissue Expression Project (GTEx), and GEO (accession: GSE17538 [40] (PMID:30606770)), where TCGA-CRC was integrated from TCGA-COAD and TCGA-READ. TCGA-CRC and GTEx expression matrices were $\log_2(\text{FPKM}+1)$ transformed and integrated, with batch effects removed using ComBat function in “sva” R package. MIF protein expression validation in normal colon and CRC tissues used The Human Protein Atlas (HPA) database (<https://www.proteinatlas.org/>); Normal/CRC tissue protein expression differential analysis was based on Clinical Proteomic Tumor Analysis Consortium (CPTAC) data via The University of Alabama at Birmingham Cancer data analysis Portal (UALCAN) (<https://ualcan.path.uab.edu/index.html>).

6 Enrichment analysis

Gene Set Variation Analysis (GSVA) was implemented using ssGSEA algorithm in “GSVA” R package. Gene Set Enrichment Analysis (GSEA) was executed via “clusterProfiler” R package. KEGG and HALLMARK gene sets originated from Molecular Signatures Database (MSigDB) (<https://www.gsea-msigdb.org/gsea/msigdb>). GO/KEGG used `enrichGO()` and `enrichKEGG()` from `clusterProfiler` for functional annotation of gene sets; Species was set to “hsa,” `pAdjustMethod`=“BH,” significance threshold $\text{FDR} < 0.05$.

7 Survival analysis

Overall survival (OS) curves and disease-specific survival (DSS) curves were plotted using “survival” and “survminer” R packages, with univariate Cox analysis performed.

8 Cell population annotation

Researchers used “FindAllMarkers” function in “Seurat” R package for cell population annotation, with cell type identification completed via “SingleR” R package [41] (PMID: 30643263) and CellMarker2.0 database [42] (PMID: 36300619). Mainly identified cell populations included: B cells (marker genes MS4A1, CD79A), T/NK cells (marker genes CD3D, CD3E, GNLY, GZMB), plasma cells (marker genes MZB1, DERL3), endothelial cells (marker genes PLVAP, VWF), fibroblasts (marker genes COL1A1, COL1A2), myeloid cells (marker genes LYZ, AIF1), mast

cells (marker genes CPA3, TPSAB1), and epithelial cells (marker genes KRT19, KRT8). Malignant epithelial cells were identified using “copykat” R package.

9 Cell interaction analysis

“CellChat” R package [43] (PMID: 33597522) was applied for cell-cell communication analysis: CellChat objects were constructed via `createCellChat` function. Interaction networks were parsed based on communication modes including “secreted signaling,” “extracellular matrix receptor,” and “direct cell contact.” Cell interaction probabilities were calculated using `computeCommunProb` function.

10 Warburg effect scores

Based on single-cell expression matrices, Warburg effect scores were calculated using ssGSEA function in Gene Set Variation Analysis (GSVA) package. Warburg effect gene set included SLC2A1, SLC2A3, HK2, PFKF, PFKFB3, ALDOA, GPI, TPI1, GAPDH, PGK1, PGAM1, ENO1, PKM, LDHA, SLC16A1, SLC16A3, PDK1 [44].

11 Tissue sample collection

Approved by Nanjing Hospital Ethics Committee affiliated to Nanjing Medical University, 21 pairs of tumor tissues and matched adjacent tissues from CRC patients were collected for subsequent MIF protein expression level detection. All patients signed written informed consent.

12 Cell culture

Human colorectal cancer (CRC) cell lines (HCT116, SW480, SW620, HT29, HCT15, LOVO, HCT8, and DLD1) and the normal intestinal epithelial cell line NCM460 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in specific ready-to-use commercial media (all obtained from KeyGEN BioTECH, Nanjing, China) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Specifically, NCM460 and HCT116 cells were cultured in DMEM; SW480 cells were cultured in Leibovitz’s L-15 medium; and SW620, HCT15, HT29, LOVO, HCT8, and DLD1 cells were cultured in RPMI-1640 medium. Cells were maintained in a humidified incubator at 37 °C with 5% CO₂. Upon reaching approximately 80% confluence, cells were passaged every 2–3 days using TrypPlus cell

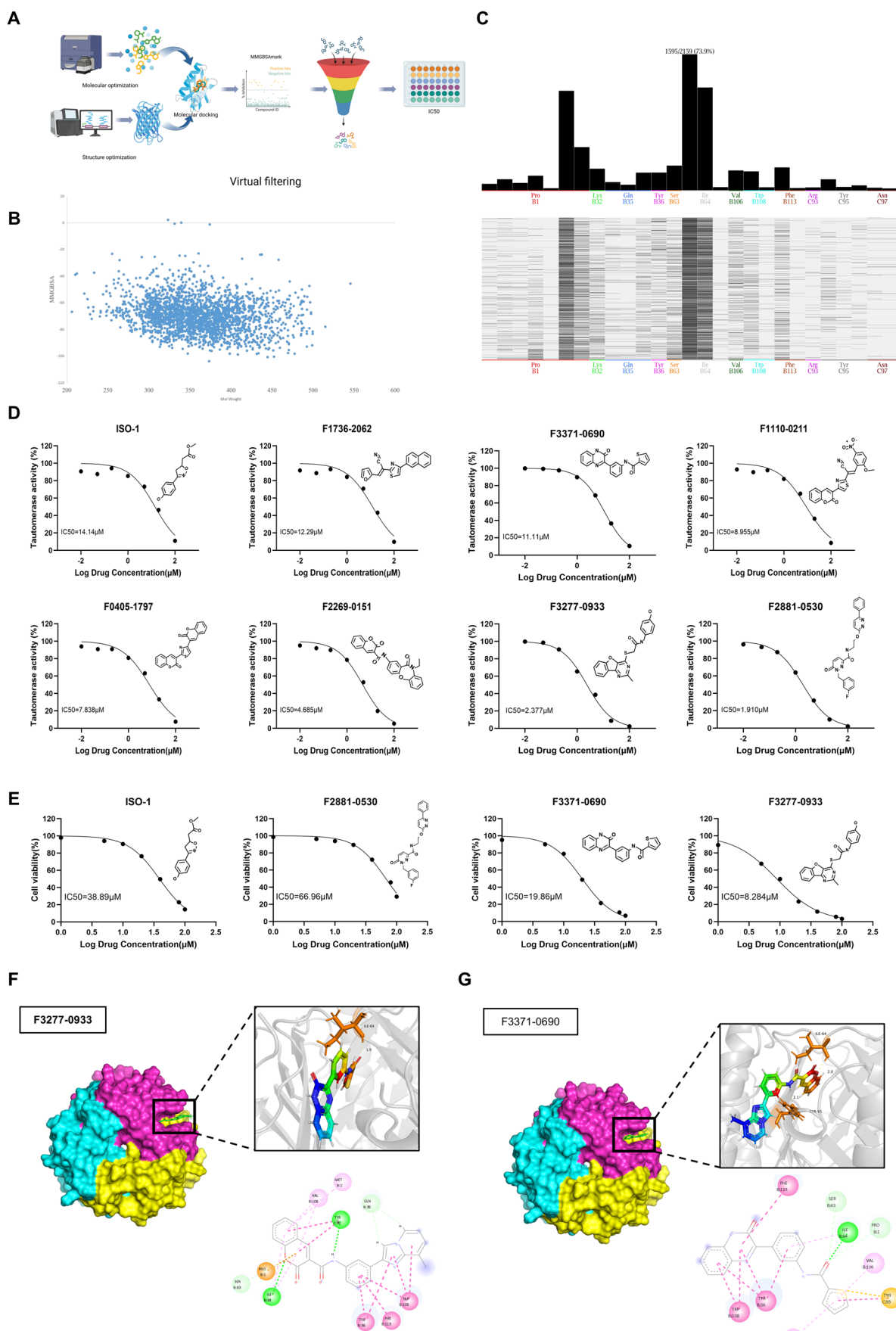


Fig. 7 Structure-Based Virtual Screening of MIF-Targeted Small-Molecule Inhibitors and Antitumor Activity Validation. **(A)** Schematic workflow of virtual screening for MIF-targeted small-molecule inhibitors. **(B)** Correlation analysis between binding free energy (MM/GBSA scores) and molecular weight of MIF candidate compounds. **(C)** Protein-ligand interaction fingerprint (PLIF) analysis for MIF. **(D)** Inhibitory effect of candidate compounds on MIF tautomerase activity (L-dopachrome methyl ester substrate-based detection). **(E)** IC50 value detection of candidate compounds inhibiting cell proliferation activity in LOVO cell lines. **(F–G)** Predicted binding modes between candidate compounds and MIF protein based on molecular docking (F3277-0933, F3371-0690). Data are presented as mean $\pm$ SD ($n=3$ independent experiments for in vitro viability assays). IC50 values were calculated using non-linear regression analysis

dissociation solution (containing EDTA and phenol red; KeyGEN BioTECH, KGL2109-100). All cell lines were authenticated via Short Tandem Repeat (STR) profiling and were confirmed to be negative for Mycoplasma contamination before being used in the experiments.

13 Protein extraction and immunoblotting

Cells were harvested using a cell scraper and lysed in RIPA lysis buffer (Beyotime, China) supplemented with protease and phosphatase inhibitor cocktails. The lysates were clarified by centrifugation at $12,000 \times g$ for 15 minutes at 4°C , and the supernatants were collected. Protein concentration was quantified by a BCA protein assay kit (KeyGEN, China). Equal amounts of protein samples were mixed with loading buffer and denatured by boiling at 95°C for 8 minutes. The proteins were then separated on 12% SDS-PAGE gels (electrophoresis conditions: 80 V for the stacking gel and 120 V for the resolving gel until the bromophenol blue dye reached the bottom) and transferred to PVDF membranes (Millipore) at a constant current of 300 mA for 60 minutes on ice. Membranes were blocked with 5% BSA for 1 hour at room temperature and sequentially incubated with primary antibodies at 4°C overnight. The specific primary antibodies used in this study were: Rabbit anti-MIF (1:1000; ABclonal, Cat# A19639), Rabbit anti-Hexokinase II (1:1000; ABclonal, Cat# A19028), Rabbit anti-LDHA (1:1000; ABclonal, Cat# A19059), and Rabbit anti-PKM2 (1:1000; ABclonal, Cat# A19074). For loading controls, Mouse anti-GAPDH (1:10000; ABclonal, Cat# AC033) and Mouse anti- β -actin (1:10000; ABclonal, Cat# AC026) were utilized. Following primary incubation, membranes were washed and incubated with HRP-conjugated Goat anti-Rabbit or Goat anti-Mouse secondary antibodies (Biosharp) at room temperature for 2 hours. Signals were developed using Feckete ultrasensitive ECL solution (Meilunbio), captured by a Bio-Rad imaging system, and band gray values were analyzed using ImageJ software. For quantitative analysis, the optical density (gray values) of the target protein

bands determined by ImageJ software was normalized to the intensity of their corresponding internal loading controls (GAPDH or β -actin) within the same sample.

14 Tissue protein extraction

Fresh tissue samples were repeatedly washed with PBS to remove impurities. Approximately 1 g tissue was weighed, minced, and placed in grinding tubes. 800 μL tissue lysis buffer (containing RIPA lysis buffer, PMSF, and phosphatase inhibitors (Beyotime)) and 3 grinding beads were added. Tissues were fully homogenized using a tissue homogenizer, centrifuged at $12,000 \times g$ for 5 minutes, and supernatants were collected as total tissue protein.

15 RNA extraction and RT-qPCR

Total RNA was extracted using FreeZol Reagent (Vazyme #R711). cDNA was synthesized with ABScript III RT Master Mix (ABclonal RK20428) under conditions: 55°C for 15 minutes $\rightarrow$ 85°C for 5 minutes. qPCR was performed using 2X Universal SYBR Green Fast qPCR Mix (ABclonal RK21203). Reaction system contained 10 μL master mix, 0.4 μL primers (10 μM), and 1–2 μL cDNA. Amplification program: 95°C pre-denaturation for 3 minutes; 40–45 cycles (95°C for 5 sec $\rightarrow$ 60°C for 30 sec); melt curve analysis. Primers for human GAPDH were purchased from Sangon Biotech (Shanghai, China; Catalog No. B661104). MIF primer sequences: Forward 5'-TCTGCCATCATGCCGATGTT-3', Reverse 5'-TTGCTGTAGGAGCGGTTCTG-3'. The relative mRNA expression levels of target genes were normalized to the endogenous reference gene (GAPDH) to correct for variations in RNA input and reverse transcription efficiency, and calculated using the $2^{-\Delta\Delta\text{Ct}}$ method with triplicate repeats.

16 Generation of stable knockout cell lines

CRISPR-Cas9 gene editing technology was used to establish stable MIF knockout (KO) cell lines. Specific sgRNA sequences targeting MIF were designed based on sgRNA prediction website (<https://portals.broadinstitute.org/gppx/gppx/crispick/public>). Designed sgRNA sequences were as follows: (5'–3') sgRNA-1-F: CACCGCACAGCATCGGCAAGATCGG, sgRNA-1-R: AAACCCGATCTTGCCGATGCTGTGC; sgRNA-2-F: CACCGTGCAGGCTGCA GAGCGCGCA, sgRNA-2-R: AAAGTGCAGGCTGCTGC AGCCTGCAC; sgRNA-3-F: CACCGGACCAGCTCAT GGCCTTCGG, sgRNA-3-R: AAACCCGAAGGCCATG

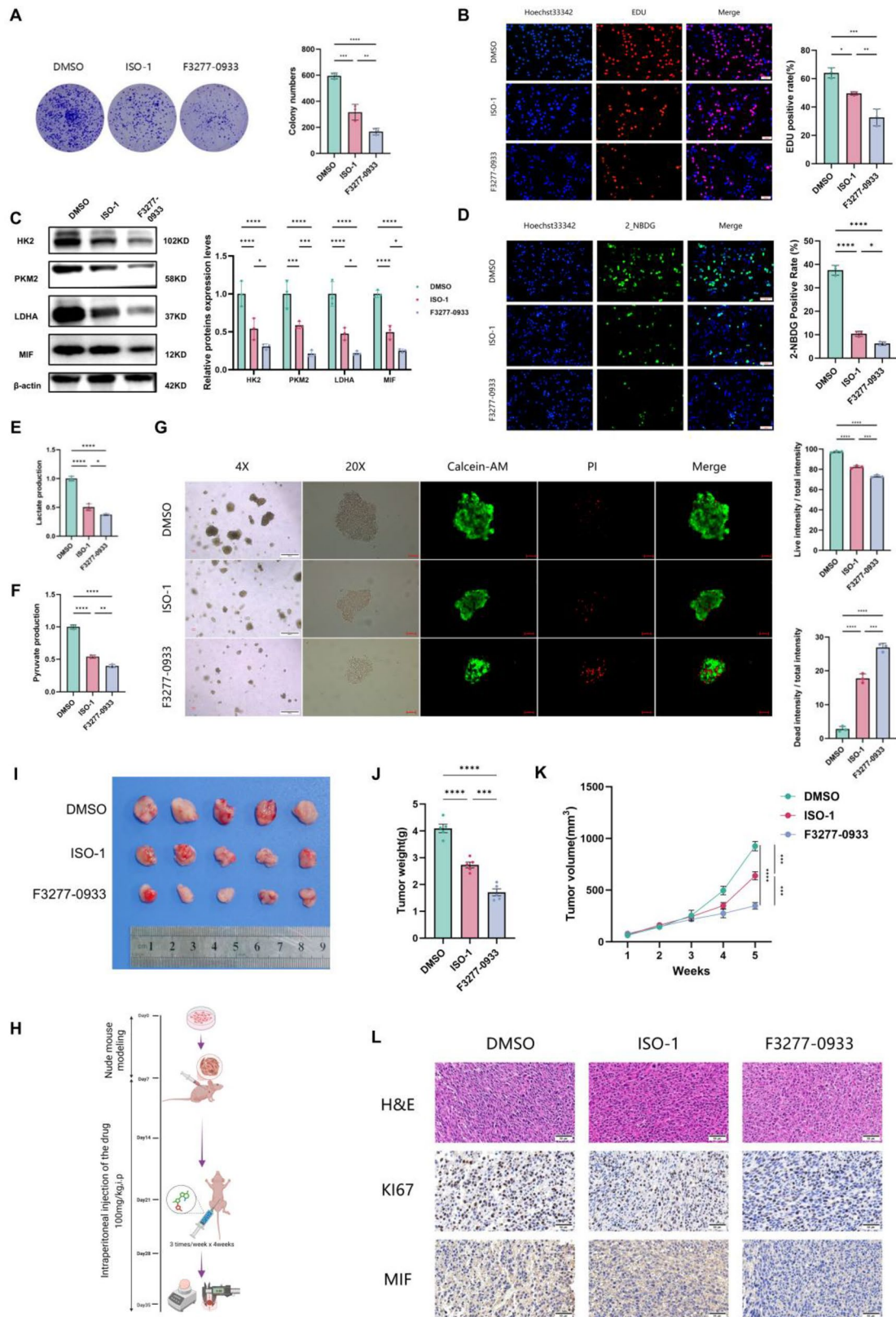


Fig. 8 The novel MIF inhibitor F3277-0933 inhibits CRC proliferation and glycolytic metabolism. **(A)** Effect of the F3277-0933 small molecule inhibitor on CRC cell colony formation ability (colony formation assay). **(B)** Effect of the F3277-0933 small molecule inhibitor on CRC cell proliferation ability (EdU assay; Scale bar=50 μ m). **(C)** Regulatory effect of the F3277-0933 small molecule inhibitor on the expression of key Warburg effect proteins (HK2, LDHA, PKM2) (Western blot analysis). **(D)** Effect of the F3277-0933 small molecule inhibitor on CRC cell glucose uptake ability (glucose uptake assay; Scale bar=50 μ m). **(E, F)** Effect of the F3277-0933 small molecule inhibitor on lactate and pyruvate production in CRC cells (lactate content assay/pyruvate content assay). **(G)** Effect of the F3277-0933 small molecule inhibitor on CRC organoids, observed microscopically after live/dead staining to assess organoid size and apoptosis (green: live cells; red: dead cells; Scale bar=50 μ m). **(H)** Schematic of the F3277-0933 small molecule inhibitor treatment experiment in tumor-bearing mice. **(I–K)** Effect of the F3277-0933 small molecule inhibitor on the in vivo tumor formation ability of CRC cells (subcutaneously-implanted xenograft model in nude mice). **(L)** Representative hematoxylin and eosin (H&E) staining and Ki67/MIF immunohistochemical (IHC) analysis of xenograft tumor tissues (Scale bar=50 μ m). Data are presented as mean $\pm$ SD ($n=3$ independent experiments for in vitro and organoid assays; $n=5$ mice per group for in vivo xenografts). Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. Exact p -values are detailed in Supplementary Table S1. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

AGCTGGTCC. Oligo DNA was synthesized and ligated into LenticrispRV2 vector. Constructed plasmids underwent Sanger sequencing to verify successful construction. Lentiviral packaging was performed in 293T cells using ExFect™ transfection reagent (Vazyme, T101). 293T cells in logarithmic growth phase were seeded in 6-well plates at 30%–40% density. When cell confluence reached 60%–80%, transfection was performed. According to optimized reagent instructions, 7 μ L ExFect reagent was mixed with 400 μ L serum-free medium (Solution A); 2.57 μ L packaging plasmids (pMD2.G, pRSV-Rev, and pMDLg/pRRE (addgene, USA) mixed at 1:1:2 ratio) and 1.43 μ g target plasmid were mixed with 400 μ L serum-free medium (Solution B). Solution A was slowly added to Solution B, gently mixed and incubated at room temperature for 15 min to form transfection complexes. 800 μ L complexes were added dropwise to cells, supplemented with 1.2 mL DMEM containing 10% FBS. Viral supernatants were collected after 48 hours and filtered through 0.22 μ m filters. Cell infection: Cells were seeded in T25 flasks. After 24 hours of culture, 2 mL viral supernatant and 5 μ g/mL polybrene (C0351, Beyotime) were added, with medium supplemented to 5 mL final volume. After 24 hours infection, complete medium containing 2 μ g/mL puromycin was replaced. Continuous screening for 2 weeks yielded stable lines.

17 Generation of stable overexpression cell lines

To generate the stable MIF-overexpressing DLD1 cell line, the full-length human MIF cDNA sequence was cloned into the lentiviral expression vector (GeneChem, Shanghai, China). Lentiviral particles were produced by co-transfecting 293T cells with the recombinant expression plasmid and the packaging plasmids (pMD2.G, pRSV-Rev, and pMDLg/pRRE) using ExFect™ transfection reagent (Vazyme) as described above. For transduction, DLD1 cells were seeded in 6-well plates at a density of 2×10^5 cells/well and incubated with viral supernatants at a multiplicity of infection (MOI) of 20 in the presence of 5 μ g/mL polybrene (GeneChem) to enhance infection efficiency. After 48 hours of incubation, the culture medium was replaced with fresh medium containing (5 μ g/mL) puromycin for 7 days to select for stably transduced cells.

18 Colony formation assay

Cells (1,500/well) were seeded in 6-well plates and cultured for 10 days. After PBS washing, cells were fixed with 4% paraformaldehyde for 15 minutes, stained with crystal violet for 15 minutes, dried, photographed, and colonies counted.

19 Transwell migration and invasion assays

Migration and invasion assays used Transwell chambers (LABSELECT 14,341). For migration assay, cells (2×10^4 /well) were resuspended in 200 μ L serum-free medium and added to upper chambers, while lower chambers contained 400 μ L medium with 10% FBS. After 24 hours, cells were fixed with 4% paraformaldehyde for 15 minutes, stained with crystal violet for 15 minutes, non-migrated cells in upper chamber removed with cotton swabs, dried, photographed under microscope, and migrated cells quantified by counting. For invasion assay, upper chambers were pre-coated with Matrigel (Corning, USA); other steps identical to migration assay.

20 Wound healing assay

Cell suspensions were prepared, and density adjusted. 5×10^5 cells were seeded per well in 6-well plates. The next day, scratches were made perpendicularly using 200 μ L pipette tip. PBS washed twice to remove detached cells. Initial observation and imaging under microscope, followed by incubation at 37 °C with 5% CO₂. After 24 hours,

images were captured at previously recorded identical positions, comparing distances between cells under different conditions.

21 EDU assay

Cells in logarithmic growth phase were seeded in 96-well plates at 2×10^4 /well density. After routine culture and treatments, 100 μ L 50 μ M EdU medium (Reagent A diluted 1:1000 in complete medium (RIBOBIO)) was added per well and incubated at 37 °C for 2 hours. Liquid was discarded, washed twice with PBS (5 min each). Fixed with 4% paraformaldehyde at room temperature for 30 minutes. Aldehyde groups blocked with glycine solution (2 mg/mL) for 5 minutes. After PBS washing, permeabilized with 0.5% Triton X-100 for 10 minutes. 100 μ L 1 $\times$ Apollo staining reaction solution was added in dark, incubated at room temperature with shaking for 30 minutes. Washed twice with PBS (10 min each). Nuclei stained with Hoechst 33,342 working solution (Reagent F diluted 1:100 in deionized water) in dark for 30 minutes. Washed three times with PBS, retaining 100 μ L PBS per well for detection.

22 BALB/c nude mouse xenograft tumor model

Xenograft tumor model evaluated MIF's role in CRC cell proliferation and tumor formation. Female BALB/c nude mice (6 weeks old, SPF grade) were used. All procedures followed institutional animal ethics guidelines. MIF-over-expressing cell line (DLD1) and MIF-knockout cell line (LOVO) were suspended in PBS (2×10^6 cells/100 μ L) and subcutaneously injected into right armpit of nude mice (5 mice per group). Tumor dimensions were measured every 7 days using a digital caliper for approximately 5 weeks. Tumor volume (V) was calculated using the formula: $V = 0.5 \times L \times W^2$, where L represents the longest diameter (length) and W represents the shortest diameter (width) perpendicular to the length. Experiment terminated when tumor volume reached $\sim 1000 \text{ mm}^3$ or mice showed distress signs. Mice were euthanized, tumors completely dissected, weighed, measured, and photographed.

23 Immunohistochemistry (IHC) and H&E staining

Detected MIF expression in CRC tissues. Tissue sections were deparaffinized with xylene and dehydrated through graded ethanol. Antigen retrieval performed with citrate

buffer (pH 6.0) under high temperature and pressure. Endogenous peroxidase activity blocked with H_2O_2 , non-specific binding blocked with 5% BSA. Sections incubated with primary antibody MIF at 4 °C overnight. Next day, after PBS washing, HRP-conjugated secondary antibody (Biosharp) applied, followed by DAB staining. Hematoxylin counter-staining performed, sections dehydrated and mounted. H&E staining: Tissue sections deparaffinized with xylene, dehydrated through graded ethanol, stained with hematoxylin for 10 minutes. After staining, rinsed under running water for ~ 10 minutes to promote blueing. Subsequently, stained with eosin, samples treated with staining solution for 2 minutes. After staining, sections dehydrated and mounted.

24 Pyruvate (PA) content detection

Pyruvate content measured using kit from Solarbio (BC2200) per manufacturer's instructions. Following 48 hours of inhibitor treatment (F3277-0933 or ISO-1) or 24 hours of Milatuzumab/rhMIF treatment, Cells cultured in T25 flasks to $\sim 80\%$ confluency, collected into centrifuge tubes. Resuspended in 1 mL extraction buffer, sonicated (ice bath, 200W power, 3 sec sonication/10 sec interval, repeated 30 times), incubated for 30 min, centrifuged at 8,000g at room temperature for 10 min. Supernatant (300 μ L) mixed with Reagent 1 (100 μ L), fully mixed, stood at room temperature for 2 min. Then 500 μ L Reagent 2 added, fully mixed. Absorbance measured at 520 nm wavelength.

25 Lactate (L-LA) content detection

Lactate content measured using kit from Solarbio (BC2230) per manufacturer's instructions. Following 48 hours of inhibitor treatment (F3277-0933 or ISO-1) or 24 hours of Milatuzumab/rhMIF treatment, Cells cultured in T25 flasks to $\sim 80\%$ confluency, collected into centrifuge tubes. Resuspended in 1 mL Extraction Buffer I, sonicated (ice bath, 300W power, 3 sec sonication/7 sec interval, repeated 18 times), centrifuged at 12,000g at room temperature for 10 min. 0.8 mL supernatant collected, slowly mixed with 0.15 mL Extraction Buffer II, gently pipetted until no bubbles. Centrifuged at 12,000g at room temperature for 10 min, supernatant collected for testing. 30 μ L supernatant mixed thoroughly with 150 μ L Reagent II working solution and 240 μ L Reagent IV working solution, reacted strictly protected from light at 37 °C for 10 min. 600 μ L Reagent V added, fully mixed. Absorbance measured at 570 nm.

26 Glucose uptake detection

Glucose uptake detected using Glucose Uptake Fluorescent Detection Kit (2-NBDG) (Beyotime, SO561S). Cells cultured in 6-well plates to ~80% confluency, then switched to glucose-free medium. After 6-hour glucose starvation, culture medium aspirated. 1 mL 2-NBDG working solution added, incubated at 37 °C in cell culture incubator for 45 min. Supernatant aspirated, washed twice with PBS. Nuclei stained with Hoechst33342 (Beyotime), incubated at room temperature protected from light for 10 min. Hoechst33342 solution aspirated, washed twice with PBS. 2 mL PBS added, observed under fluorescence microscope.

27 Measurement of cellular glycolysis and mitochondrial respiration

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies, USA) to evaluate mitochondrial oxidative phosphorylation (OXPHOS) and glycolytic function, respectively. For the mitochondrial respiration assay, cells were seeded in Seahorse XF96 cell culture microplates at a density of 1.5×10^4 cells/well and cultured overnight. Cells were then treated with F3277-0933 or ISO-1 for 48 hours, or with Milatuzumab/rhMIF for 24 hours. On the day of the assay, the culture medium was replaced with Seahorse XF base medium supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine (pH 7.4). Cells were equilibrated in a non-CO₂ incubator at 37 °C for 1 hour prior to the assay. During the measurement, modulators from the Mitochondrial Stress Test Kit were injected sequentially to reach the following final concentrations: Oligomycin (1.0 μM), FCCP (1.0 μM), and Rotenone/Antimycin A (0.5 μM). For the glycolysis assay, the culture medium was replaced with glucose-free Seahorse XF base medium supplemented with 2 mM glutamine (pH 7.4). Cells were equilibrated in a non-CO₂ incubator at 37 °C for 1 hour. Reagents from the Glycolysis Stress Test Kit were injected sequentially to reach the following final concentrations: Glucose (10 mM), Oligomycin (1.0 μM), and 2-Deoxy-D-glucose (2-DG, 50 mM). OCR and ECAR values were recorded using Wave software (Agilent Technologies) and normalized to total cellular protein content determined by the BCA assay immediately post-assay. Each biological sample was measured in technical triplicates.

28 Cell treatments

For in vitro functional blockade and rescue experiments, colorectal cancer cells were seeded in appropriate multi-well plates and allowed to adhere overnight. To block the CD74 signaling pathway, cells were pre-treated with the anti-CD74 neutralizing antibody Milatuzumab (MedChem-Express, Cat# HY-P99731) at a concentration of 5 μg/mL for 24 hours. For rescue experiments, cells were stimulated with recombinant human MIF (rhMIF; Abclonal, Cat# RP02895) at a concentration of 100 ng/mL for 24 hours prior to subsequent analysis. For inhibitor efficacy evaluation, cells were treated with F3277-0933 or ISO-1 at their respective designated concentrations for 48 hours before harvesting for protein extraction or metabolite quantification.

29 Virtual screening of small-molecule inhibitors targeting MIF protein

Target protein preprocessing: Based on MIF crystal structure (PDB ID: 7XTX), structure optimization performed using Protein Preparation Wizard module in Schrödinger software, including bond order correction, hydrogen atom addition, disulfide bond assignment. Protonation at pH 7.0 using PROPKA method, constrained energy optimization via OPLS4 force field (heavy atom RMSD convergence at 0.3 Å). Compound library preparation: LF1000 database processed by LigPrep module, protonated/deprotonated at pH 7.0 ± 2.0 using Epik method, desalted, original chirality retained, generating up to 32 conformations per molecule. Hierarchical virtual screening: First, standard precision (SP) docking retained top 20% scoring molecules; Then, extra precision (XP) docking performed secondary screening retaining top 20%; Finally, binding free energy calculated via MMGBSA for affinity assessment. Result filtering and verification: Molecules forming no protein interactions excluded (172), retaining 2159 candidate molecules. Molecules filtered based on MMGBSA energy (< -80 kcal/mol), clustered by 70% structural similarity using MOE software, yielding 308 representative compound classes. Binding modes verified via PLIF interaction fingerprint analysis (key residues ILE64/PRO1) and visualization [45, 46].

30 Small-molecule inhibitor IC₅₀ detection

Well-grown CRC cells digested with trypsin, uniformly seeded in 48-well plates (cultured at 37 °C, 5% CO₂ for 24 hours). Eight drug concentration gradients set (0, 1, 5, 10, 20, 40, 80, 100 μM), with three replicate wells per concentration. Next day, medium replaced with corresponding

drug-containing medium, refreshed every 2 days to maintain drug concentration. After 7 days culture, experiment terminated. Fixed with 4% paraformaldehyde at room temperature for 15 min, washed 3 times with PBS (5 min each). Stained with crystal violet solution at room temperature for 10 min, washed 3 times with PBS, photographed. 200 μ L 2% SDS added per well to dissolve dye, incubated on shaker for 30 min. Transferred to 96-well plate, 60 μ L per well from three replicate wells. Absorbance detected at 570 nm wavelength using multifunctional microplate reader.

31 Tissue sample collection and organoid establishment

Tumor specimens were obtained from two consenting patients with colorectal cancer (CRC) to establish patient-derived organoids (PDOs). Patient 1 was a 65-year-old female diagnosed with adenocarcinoma of the ascending colon. Postoperative pathological staging confirmed a stage of pT3N1b. Patient 2 was a 72-year-old male also diagnosed with adenocarcinoma of the ascending colon, presenting with lymph node metastasis. His postoperative pathological staging was confirmed as pT3N2a. The histological diagnosis and staging were validated by pathologists in accordance with the AJCC Cancer Staging Manual (8th Edition). All procedures were approved by the Ethics Committee of Nanjing First Hospital, and written informed consent was obtained from each patient.

32 Organoid culture

Sample processing: Fresh tumor tissue placed in ice-cold DMEM/F12 with 1% penicillin-streptomycin-gentamicin. After transfer, impurities removed, cut into 3–5 mm³ fragments. Washed 10 times with ice-cold DPBS (with antibiotics), further dissociated into 1 mm³ fragments. **Digestion and filtration:** 5–10 mL tumor digestion solution added, shaken at 37 °C, 120 rpm for 20 min. Digestion terminated when single cells/cell clusters observed microscopically. Filtered through 70 μ m strainer, centrifuged at 300 $\times$ g 4 °C for 5 min. Washed twice with DMEM/F12. **Inoculation culture:** Precipitate mixed with Matrigel at 1:5 volume ratio, vertically inoculated (30 μ L/well), gelled at 37 °C for 20 min. Human intestinal organoid medium added (500 μ L/well), cultured at 37 °C, 5% CO₂, medium changed every 3 days. The patient-derived organoids (PDOs) were cultured using a defined, serum-free bioGenous™ Colorectal Cancer Organoid Kit (Catalog: K2103-CR, bioGenous TECHNOLOGIES). The complete organoid medium was prepared precisely according to the manufacturer's instructions.

33 Organoid live/dead staining

Detected using Calcein-AM/PI Live/Dead Double Stain Kit (Solarbio, CA1630). First, well-grown CRC organoids gently mixed in Matrigel containing 10 μ M inhibitor (or equal volume DMSO), inoculated in culture plates, placed at 37 °C, 5% CO₂ incubator for 20 minutes for Matrigel solidification. 500 μ L human intestinal organoid medium containing same concentration inhibitor (or equal volume DMSO) added per well. Medium containing inhibitor (or DMSO) changed every three days with morphological changes observed. Before staining, 10 $\times$ Assay Buffer equilibrated to room temperature and diluted 1:9 with ddH₂O to 1 $\times$ Assay Buffer. Staining started: Medium discarded, DPBS added and stood for 3 minutes. Gently pipetted, centrifuged at 300 g for 5 minutes to collect organoids in 1.5 mL centrifuge tubes. Washed once with DPBS (300 g centrifugation 5 minutes) to remove Matrigel. Organoids resuspended in 1 $\times$ Assay Buffer, 2 μ L Calcein-AM stock solution added per mL suspension, gently flick-mixed. Incubated at 37 °C protected from light for 20 minutes. 5 μ L PI stock solution added, stained at room temperature protected from light for 5 minutes. Centrifuged at 450 g for 5 minutes, supernatant discarded. Washed with 1 mL PBS (450 g centrifugation 5 minutes). Finally, organoid pellet mixed with appropriate amount of antifade mounting medium, gently flick-mixed. 3 μ L suspension dropped on slide, observed under 490 $\pm$ 10 nm excitation light: Yellow-green fluorescence (Calcein) indicates live cells; red fluorescence (PI) indicates dead cells.

34 Enzyme activity detection method

MIF tautomerase activity assay used L-dopachrome methyl ester substrate-based method, quantifying enzyme activity inhibition by real-time monitoring absorbance changes of oxidized products. First, 6 mM L-3,4-dihydroxyphenylalanine methyl ester hydrochloride (Sigma) and 12 mM sodium metaperiodate (Sigma) mixed in equal volumes to prepare fresh L-dopachrome methyl ester substrate. Subsequently, test compounds and rhMIF (Abclonal, RP01829) were pre-incubated for 30 minutes at room temperature in 10 mM potassium phosphate buffer (pH 6.2) containing 0.5 mM EDTA buffer system. 120 μ L pre-incubation mixture and 30 μ L substrate added to 96-well plate, immediately monitoring absorbance changes at 475 nm wavelength (15 sec intervals, lasting 5 minutes). Enzyme activity calculated with IC₅₀ values relative to untreated control group (DMSO group) as 100% benchmark [28, 46].

35 BALB/c nude mouse tumor-bearing model construction and drug treatment

For the in vivo efficacy evaluation, 2×10^6 LOVO cells were subcutaneously injected into the right axilla of 6-week-old male BALB/c nude mice. After 7 days, when the average tumor volume reached approximately 0.1 cm^3 , the mice were randomly assigned to different treatment groups. The small-molecule inhibitors (F3277-0933 and ISO-1) were formulated in a vehicle consisting of 10% DMSO and 90% physiological saline. Mice in the treatment groups were administered the compounds intraperitoneally (i.p.) at a dose of 100 mg/kg body weight, three times a week for 4 weeks. To rigorously control for solvent effects, mice in the vehicle control group (DMSO group) received an equal volume of the exact same solvent mixture (10% DMSO and 90% physiological saline) via the same injection route. Tumor volumes and body weights were recorded prior to each administration.

36 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA) and R software version 4.3.1. Data are presented as mean $\pm$ standard deviation (SD) from a minimum of three independent biological replicates. To properly assign statistical tests to their corresponding analyses, the following approaches were utilized: For continuous variables with normal distribution, comparisons between two independent groups were analyzed using the unpaired Student's *t*-test (e.g., in vitro functional assays and xenograft tumor weights), while comparisons between matched clinical tumor/adjacent tissues were performed using the paired Student's *t*-test. For non-parametric data, such as single-cell RNA sequencing differential expression, the Wilcoxon rank-sum test (Mann-Whitney U test) was applied. For comparisons among three or more groups (e.g., rescue experiments and multiple inhibitor treatments), a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed. Patient survival outcomes were evaluated using the Kaplan-Meier method, and statistical significance was determined by the Log-rank test. IC₅₀ values for small-molecule inhibitors were calculated utilizing non-linear regression analysis. The statistical significance level was set at $p < 0.05$. Significance thresholds were defined as: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Exact *p*-values are provided in the text and figure legends where applicable, except for values less than 0.0001, which are reported as $p < 0.0001$. A comprehensive list of exact *p*-values for all

relevant statistical comparisons is provided in Supplementary Table S1.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13402-026-01202-9>.

Author contributions JL and YC analyzed all data from this study and completed the manuscript. JL, YC, JL, MD and ZL performed the in vitro experiments. HS, JQ, and JL was responsible for the collection of clinical samples. YP and HS designed the idea of the study and examined and revised the manuscript. JL and YC contributed equally to this work. All authors read and approved the final manuscript.

Funding This work was supported by grants from the Jiangsu Provincial Medical Key Discipline Cultivation Unit (JSDW202239), Project of Science and Technology Development of Nanjing Medicine (YKK24130), Industry-University-Research Innovation Fund for Chinese Universities -digital health project (2023GY014), Specialized Cohort Research Project of Nanjing Medical University (NMUC2020035, NMUC2021013A).

Data availability The datasets supporting the conclusions of this article are included within the article.

Declarations

Ethics approval and consent to participate This research was performed in compliance with the ethical standards outlined in the Declaration of Helsinki. The studies involving human participants were reviewed and approved by the Ethics Committees and Institutional Review Boards of Nanjing First Hospital, affiliated with Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Consent for publication All authors have provided their consent for publication.

Competing interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

References

1. R.L. Siegel, T.B. Kratzer, A.N. Giaquinto, H. Sung, A. Jemal, Cancer statistics, 2025. *CA Cancer J. Clin.* **75**(1), 10–45 (2025). <https://doi.org/10.3322/caac.21871>
2. F. Wang, G. Chen, Z. Zhang, Y. Yuan, Y. Wang, Y.H. Gao, W. Sheng, Z. Wang, X. Li, X. Yuan, S. Cai, L. Ren, Y. Liu, J. Xu, Y. Zhang, H. Liang, X. Wang, A. Zhou, J. Ying, G. Li, M. Cai, G. Ji, T. Li, J. Wang, H. Hu, K. Nan, L. Wang, S. Zhang, J. Li, R.-H. Xu, The Chinese society of clinical oncology (CSCO): clinical guidelines for the diagnosis and treatment of colorectal cancer, 2024 update. *Cancer Commun.* **45**(3), 332–379 (2025). <https://doi.org/10.1002/cac2.12639>
3. Q. Li, S. Geng, H. Luo, W. Wang, Y.Q. Mo, Q. Luo, L. Wang, G.B. Song, J.P. Sheng, B. Xu, Signaling pathways involved in colorectal cancer: pathogenesis and targeted therapy. *Sig Transduct Target Ther.* **9**(1), 266 (2024). <https://doi.org/10.1038/s41392-024-01953-7>
4. E. Dekker, P.J. Tanis, J.L.A. Vleugels, P.M. Kasi, M.B. Wallace, Colorectal cancer. *Lancet* **394**(10207), 1467–1480 (2019). [https://doi.org/10.1016/S0140-6736\(19\)32319-0](https://doi.org/10.1016/S0140-6736(19)32319-0)
5. L. Leng, C.N. Metz, Y. Fang, J. Xu, S. Donnelly, J. Baugh, T. Delohery, Y. Chen, R.A. Mitchell, R. Bucala, MIF Signal transduction initiated by binding to CD74. *J. Exp. Med.* **197**(11), 1467–1476 (2003). <https://doi.org/10.1084/jem.20030286>
6. M.D. Sanchez-Niño, A.B. Sanz, O. Ruiz-Andres, J. Poveda, M.C. Izquierdo, R. Selgas, J. Egido, A. Ortiz, MIF, CD74 and other partners in kidney disease: tales of a promiscuous couple. *Cytokine Growth Factor Rev.* **24**(1), 23–40 (2013). <https://doi.org/10.1016/j.cytogfr.2012.08.001>
7. K. Sumaiya, D. Langford, K. Natarajaseenivasan, S. Shanmughapriya, Macrophage migration inhibitory factor (MIF): a multifaceted cytokine regulated by genetic and physiological strategies. *Pharmacol. Ther.* **233**, 108024 (2022). <https://doi.org/10.1016/j.pharmthera.2021.108024>
8. X. Shi, L. Leng, T. Wang, W. Wang, X. Du, J. Li, C. McDonald, Z. Chen, J.W. Murphy, E. Lolis, P. Noble, W. Knudson, R. Bucala, CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity* **25**(4), 595–606 (2006). <https://doi.org/10.1016/j.immuni.2006.08.020>
9. Y. Gore, D. Starlets, N. Maharshak, S. Becker-Herman, U. Kaneyuki, L. Leng, R. Bucala, I. Shachar, Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex. *J. Biol. Chem.* **283**(5), 2784–2792 (2008). <https://doi.org/10.1074/jbc.M703265200>
10. H. Lue, M. Dewor, L. Leng, R. Bucala, J. Bernhagen, Activation of the JNK signalling pathway by macrophage migration inhibitory factor (MIF) and dependence on CXCR4 and CD74. *Cell. Signal.* **23**(1), 135–144 (2011). <https://doi.org/10.1016/j.cellsig.2010.08.013>
11. X.-X. He, K. Chen, J. Yang, X.-Y. Li, H.-Y. Gan, C.-Y. Liu, T.R. Coleman, Y. Al-Abed, Macrophage migration inhibitory factor promotes colorectal cancer. *Mol. Med.* **15**(1–2), 1–10 (2009). <https://doi.org/10.2119/molmed.2008.00107>
12. S. Hu, J. Feng, W. Fu, Y. Guo, Macrophage migration inhibitory factor (MIF) upregulates CXCR7 and contributes to chemotherapy resistance in colorectal cancer. *Cell. Biochem. Biophys.* **82**(4), 3437–3452 (2024). <https://doi.org/10.1007/s12013-024-01430-6>
13. L. Yan, M. Wu, T. Wang, H. Yuan, X. Zhang, H. Zhang, T. Li, V. Pandey, X. Han, P.E. Lobie, T. Zhu, Breast cancer stem cells secrete MIF to mediate tumor metabolic reprogramming that drives immune evasion. *Cancer Res.* **84**(8), 1270–1285 (2024). <https://doi.org/10.1158/0008-5472.CAN-23-2390>
14. Q. Cui, S. Li, X. Liu, J. Liu, W. Chen, Y. Sheng, P. Xie, L. Jin, F. Zeng, F. Lv, X. Hu, R.-P. Xiao, MIF-ACKR3 causes irreversible fat loss by impairing adipogenesis in cancer cachexia. *Cell. Metab.* **37**(4), 954–970.e8 (2025). <https://doi.org/10.1016/j.cmet.2025.01.018>
15. P.V. Tilstam, W. Schulte, T. Holowka, B.S. Kim, J. Nouws, M. Sauler, M. Piecychna, G. Pantouris, E. Lolis, L. Leng, J. Bernhagen, G. Fingerle-Rowson, R. Bucala, MIF but not MIF-2 recruits inflammatory macrophages in an experimental polymicrobial sepsis model. *J. Clin. Investigation* **131**(23 2021). <https://doi.org/10.1172/JCI127171>
16. M.T. Bozza, L. Lintomen, J.Z. Kitoko, C.N. Paiva, P.C. Olsen, The role of MIF on eosinophil biology and eosinophilic inflammation. *Clinic Rev Allerg Immunol.* **58**(1), 15–24 (2020). <https://doi.org/10.1007/s12016-019-08726-z>
17. Q. Wang, D. Zhao, M. Xian, Z. Wang, E. Bi, P. Su, J. Qian, X. Ma, M. Yang, L. Liu, Y. Zu, S.R. Pingali, K. Chen, Z. Cai, Q. Yi, MIF as a biomarker and therapeutic target for overcoming resistance to proteasome inhibitors in human myeloma. *Blood* **136**(22), 2557–2573 (2020). <https://doi.org/10.1182/blood.2020005795>
18. C. O'Reilly, M. Doroudian, L. Mawhinney, S.C. Donnelly, Targeting MIF in cancer: therapeutic strategies, current developments, and future opportunities. *Med Res Rev.* **36**(3), 440–460 (2016). <https://doi.org/10.1002/med.21385>
19. Y. Al-Abed, S. VanPatten, MIF as a disease target: ISO-1 as a proof-of-concept therapeutic. *Future medicinal chemistry. Future Med Chem.* **3**(1), 45–63 (2011). <https://doi.org/10.4155/fmc.10.281>
20. J. Jarmula, J. Lee, A. Lauko, P. Rajappa, M.M. Grabowski, A. Dhawan, P. Chen, R. Bucala, M.A. Vogelbaum, J.D. Lathia, Macrophage migration inhibitory factor as a therapeutic target in neuro-oncology: a review. *Neuro-Oncol. Adv.* **6**(1 2024). <https://doi.org/10.1093/oaajnl/vdae142>
21. L. Putha, L.K. Kok, M. Fellner, M.T. Rutledge, A.B. Gamble, S.M. Wilbanks, A.J. Vennall, J.D.A. Tyndall, Covalent isothiocyanate inhibitors of Macrophage migration inhibitory factor as potential colorectal cancer treatments. *ChemMedChem* **19**(21 2024). <https://doi.org/10.1002/cmdc.202400394>
22. W.H. Koppenol, P.L. Bounds, C.V. Dang, Otto Warburg's contributions to current concepts of cancer metabolism. *Nat. Rev. Cancer.* **11**(5), 325–337 (2011). <https://doi.org/10.1038/nrc3038>
23. F. Fontana, G. Giannitti, S. Marchesi, P. Limonta, The PI3K/Akt pathway and glucose metabolism: a dangerous liaison in cancer. *Int. J. Biol. Sci.* **20**(8), 3113–3125 (2024). <https://doi.org/10.7150/ijbs.89942>
24. P. Icard, S. Shulman, D. Farhat, J.M. Steyaert, M. Alifano, H. Lincet, How the Warburg effect supports aggressiveness and drug resistance of cancer cells? *Drug resistance updates: reviews and commentaries in antimicrobial and anticancer chemotherapy. Drug. Resist. Updat.* **38**, 1–11 (2018). <https://doi.org/10.1016/j.drup.2018.03.001>
25. Z. Wang, Q. Jiang, C. Dong, Metabolic reprogramming in triple-negative breast cancer. *Cancer Biol. Med.* **17**(1), 44–59 (2020). <https://doi.org/10.20892/j.issn.2095-3941.2019.0210>
26. J.A. Enríquez, M. Mittelbrunn, M. Mittelbrunn, Warburg effect reshapes tumor immunogenicity. *Cancer Res.* **84**(13), 2043–2045 (2024). <https://doi.org/10.1158/0008-5472.CAN-24-1304>
27. S.M. Fendt, 100 years of the Warburg effect: a cancer metabolism endeavor. *Cell* **187**(15), 3824–3828 (2024). <https://doi.org/10.1016/j.cell.2024.06.026>
28. L. Xu, Y. Zhang, L. Zheng, C. Qiao, Y. Li, D. Li, X. Zhen, T. Hou, Discovery of novel inhibitors targeting the macrophage migration inhibitory factor via structure-based virtual screening and bioassays. *J. Med. Chem.* **57**(9), 3737–3745 (2014). <https://doi.org/10.1021/jm401908w>

29. I. Kang, R. Bucala, The immunobiology of MIF: function, genetics and prospects for precision medicine. *Nat. Rev. Rheumatol.* **15**(7), 427–437 (2019). <https://doi.org/10.1038/s41584-019-0238-2>
30. A.C. Gonçalves, E. Richiandone, J. Jorge, B. Polónia, C.P.R. Xavier, I.C. Salaroglio, C. Riganti, M.H. Vasconcelos, C. Corbet, A.B. Sarmiento-Ribeiro, Impact of cancer metabolism on therapy resistance – clinical implications. *Drug. Resist. Updat.* **59**, 100797 (2021). <https://doi.org/10.1016/j.drug.2021.100797>
31. N.N. Pavlova, J. Zhu, C.B. Thompson, The hallmarks of cancer metabolism: still emerging. *Cell. Metab.* **34**(3), 355–377 (2022). <https://doi.org/10.1016/j.cmet.2022.01.007>
32. J. Feng, J. Li, L. Wu, Q. Yu, J. Ji, J. Wu, W. Dai, C. Guo, Emerging roles and the regulation of aerobic glycolysis in hepatocellular carcinoma. *J. Exp. Clin. Cancer Res.* **39**(1), 126 (2020). <https://doi.org/10.1186/s13046-020-01629-4>
33. H. Chen, Y. Li, H. Li, X. Chen, H. Fu, D. Mao, W. Chen, L. Lan, C. Wang, K. Hu, J. Li, C. Zhu, I. Evans, E. Cheung, D. Lu, Y. He, A. Behrens, D. Yin, C. Zhang, NBS1 lactylation is required for efficient DNA repair and chemotherapy resistance. *Nature* **631**(8021), 663–669 (2024). <https://doi.org/10.1038/s41586-024-07620-9>
34. W. Zhang, M. Xia, J. Li, G. Liu, Y. Sun, X. Chen, J. Zhong, Warburg effect and lactylation in cancer: mechanisms for chemoresistance. *Mol. Med.* **31**(1), 146 (2025). <https://doi.org/10.1186/s10020-025-01205-6>
35. B. Polónia, C.P.R. Xavier, J. Kopecka, C. Riganti, M.H. Vasconcelos, The role of extracellular vesicles in glycolytic and lipid metabolic reprogramming of cancer cells: consequences for drug resistance. *Cytokine Growth Factor Rev.* **73**, 150–162 (2023). <https://doi.org/10.1016/j.cytogfr.2023.05.001>
36. E. Lionta, G. Spyrou, D.K. Vassilatis, Z. Cournia, Cournia Z: structure-based virtual screening for drug discovery: principles, applications and recent advances. *CTMC* **14**(16), 1923–1938 (2014). <https://doi.org/10.2174/1568026614666140929124445>
37. H.O. Lee, Y. Hong, H.E. Etlioglu, Y.B. Cho, V. Pomella, B. Van den Bosch, J. Vanhecke, S. Verbandt, H. Hong, J.W. Min, N. Kim, H.H. Eum, J. Qian, B. Boeckx, D. Lambrechts, P. Tsantoulis, G. De Hertogh, W. Chung, T. Lee, M. An, H.-T. Shin, J.-G. Joong, M.-H. Jung, G. Ko, P. Wirapati, S.H. Kim, H.C. Kim, S.H. Yun, I.B.H. Tan, B. Ranjan, W.Y. Lee, T.-Y. Kim, J.K. Choi, Y.-J. Kim, S. Prabhakar, S. Tejpar, W.-Y. Park, Lineage-dependent gene expression programs influence the immune landscape of colorectal cancer. *Nat. Genet.* **52**(6), 594–603 (2020). <https://doi.org/10.1038/s41588-020-0636-z>
38. F. Uhlitz, P. Bischoff, S. Peidli, A. Sieber, A. Trinks, M. Lüthen, B. Obermayer, E. Blanc, Y. Ruchiy, T. Sell, S. Mamlouk, R. Arsie, T.-T. Wei, K. Klotz-Noack, R.F. Schwarz, B. Sawitzki, C. Kamphues, D. Beule, M. Landthaler, C. Sers, D. Horst, N. Blüthgen, M. Morkel, Mitogen-activated protein kinase activity drives cell trajectories in colorectal cancer. *EMBO Mol Med.* **13**(10), e14123 (2021). <https://doi.org/10.15252/emmm.202114123>
39. I. Korsunsky, N. Millard, J. Fan, K. Slowikowski, F. Zhang, K. Wei, Y. Baglaenko, M. Brenner, P.R. Loh, S. Raychaudhuri, Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat Methods* **16**(12), 1289–1296 (2019). <https://doi.org/10.1038/s41592-019-0619-0>
40. M.S. Chen, Y.H. Lo, X. Chen, C.S. Williams, J.M. Donnelly, Criss ZK, 2nd, Z.K. Criss, S. Patel, J. Butkus, M.J. Dubrulle, M.J. Finegold, N.F. Shroyer, Growth factor-independent 1 is a tumor suppressor gene in colorectal cancer. *Mol. Cancer Res.* **17**(3), 697–708 (2019). <https://doi.org/10.1158/1541-7786.MCR-18-0666>
41. D. Aran, A.P. Looney, L. Liu, E. Wu, V. Fong, A. Hsu, S. Chak, R.P. Naikawadi, P.J. Wolters, A.R. Abate, A.J. Butte, M. Bhattacharya, Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage. *Nat. Immunol.* **20**(2), 163–172 (2019). <https://doi.org/10.1038/s41590-018-0276-y>
42. C. Hu, T. Li, Y. Xu, X. Zhang, F. Li, J. Bai, J. Chen, W. Jiang, K. Yang, Q. Ou, X. Li, P. Wang, Y. Zhang, CellMarker 2.0: an updated database of manually curated cell markers in human/mouse and web tools based on scRNA-seq data. *Nucleic Acids Res.* **51**(D1), D870–d876 (2023). <https://doi.org/10.1093/nar/gka c947>
43. S. Jin, C.F. Guerrero-Juarez, L. Zhang, I. Chang, R. Ramos, C.H. Kuan, P. Myung, M.V. Plikus, Q. Nie, Inference and analysis of cell-cell communication using CellChat. *Nat Commun.* **12**(1), 1088 (2021). <https://doi.org/10.1038/s41467-021-21246-9>
44. M.G. Vander Heiden, L.C. Cantley, C.B. Thompson, Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Sci. (New Y., NY)* **324**(5930), 1029–1033 (2009). <https://doi.org/10.1126/science.1160809>
45. Z. Cournia, L. Leng, S. Gandavadi, X. Du, R. Bucala, W.L. Jorgensen, Discovery of human Macrophage migration inhibitory factor (MIF)-CD74 Antagonists via virtual screening. *J. Med. Chem.* **52**(2), 416–424 (2009). <https://doi.org/10.1021/jm801100v>
46. Y. Zhang, L. Xu, Y. Zhang, J. Pan, P.-Q. Wang, S. Tian, H.-T. Li, B.-W. Gao, T.-J. Hou, X.-C. Zhen, L.-T. Zheng, Discovery of novel MIF inhibitors that attenuate microglial inflammatory activation by structures-based virtual screening and in vitro bioassays. *Acta. Pharmacol. Sin.* **43**(6), 1508–1520 (2022). <https://doi.org/10.1038/s41401-021-00753-x>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.